DEC 26 1922

Vol. III. No. 6] [December, 1922.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C. 1.
CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, post free, Two Pounds net.
Single Numbers, Seven and Sixpence net.

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THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

A NEW MEDIUM FOR THE ISOLATION OF *B. DIPHTHERIÆ.*

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Received for publication August 6th, 1922.

THE reasons which led to this medium being employed were as follows: The work initiated by Wright (1915), and amplified by Flemming, Colebrook and myself (1920), in which it was shown that the complete or partial neutralisation of the anti-tryptic power enormously increased the value of serum as a medium, suggested that if a suitable proportion of such a trypsin and serum mixture was added to agar the growth of *B. diphtheriæ* might be greatly increased. It was also thought that these conditions were in all probability those that obtained in the false membrane where the bacteria grew so freely. Preliminary trials were therefore made in which the growth of *B. diphtheriæ* on media prepared by adding varying percentages of trypsinised serum to agar was compared with that on agar to which a corresponding amount of untreated serum had been added, with the result that media containing trypsinised serum showed itself in every way superior. This medium had, however, two disadvantages: (1) It was difficult to recognise the colonies of *B. diphtheriæ* from those of other organisms; (2) many of the organisms commonly associated with *B. diphtheriæ* in throat swabs also grew very luxuriantly. Following the work of Conradi and Troch (1912), and J. F. Smith (1914), it was decided to add a percentage of potassium tellurite in hopes of checking the growth of the associated organisms and also of giving a distinctive appearance to the colonies of *B. diphtheriæ.*

PREPARATION OF THE MEDIUM.

Reagents required: (*a*) Nutrient agar; (*b*) solution of potassium tellurite 1 per cent.; (*c*) sterile trypsinised serum.

(*a*) The nutrient agar preferably employed is one made with broth prepared by digesting meat with trypsin, as described by me (1914), but ordinary agar made with peptone broth gives almost identical results. Two per cent. of agar should be dissolved in the broth.

(*b*) The solution of potassium tellurite is somewhat troublesome to prepare, as a proportion of the salt usually fails to dissolve. This difficulty can be

surmounted by dissolving as much as possible of the salt in a proportion of the distilled water, allowing the insoluble portion to settle, and then pouring off the clear, supernatant layer as completely as possible. The deposit is rubbed up with a solution of 10 per cent. potassium hydrate, added drop by drop, until completely dissolved. In order to neutralise the excess of alkali, hydrochloric acid is cautiously added until a precipitate commences to appear. This fraction is then added to the portion of the salt which readily dissolved, and the solution is made up to the requisite volume with distilled water. Once dissolved the solution remains clear for a considerable period, if kept in a vessel securely corked.

(*c*) Sterile trypsinised serum. This consists of serum to which sufficient trypsin has been added to neutralise partially the antitryptic power.

It may be prepared either (1) by adding a sterile sample of trypsin to sterile serum, or (2) by sterilising the mixture of serum and trypsin by filtration.

(1) Sterile serum—horse-serum is usually employed—is procured either by bleeding the animal, with aseptic precautions, or by adding 0·25 per cent. of chloroform to serum obtained from the slaughter-house and carefully freed from red blood-corpuscles. This mixture is then placed in a tightly glass-stoppered bottle and kept at a moderately high temperature, such as 37° C., or better 42° C., for a few days. Sterile glycerinated solutions of trypsin may be obtained in sealed glass ampoules either from Messrs. Fairchild & Co., or from the Digestive Ferments Co., U.S.A. Both these products possess a similar tryptic power, and appear reliable as regards sterility.

To neutralise the antitryptic power of 100 c.c. serum from a normal horse rather more than 5 c.c. of either of these products is necessary, but for the present purpose the addition of 2 to 4 c.c. of the sterile trypsin to 100 c.c. of the sterile serum is sufficient. Such a mixture keeps for months, and is best stored in quantities sufficient for making up a single batch of medium.

An easy method of testing the strength of a trypsin solution is as follows: Fresh milk is taken and the cream removed by centrifuging. Sufficient calcium chloride solution is then added to bring the concentration up to 1 per cent.

Graded dilutions of the trypsin are then made, and an equal volume of each dilution and of the milk are mixed together in a series of dwarf test-tubes. The tubes are placed in a water-bath at 50° C. for 15 or 30 minutes, after which they are examined to ascertain the highest dilution of the trypsin which causes clotting of the milk. This method is founded on the observation of Mellanby and Woolley (1912–13), that trypsin caused the clotting of milk to which calcium chloride had been added.

When it is desired to ascertain the antitryptic power of a serum, the following mixtures are made in a series of dwarf test-tubes: one volume of milk containing 1 per cent. of calcium chloride; one volume of serum; one volume of graded dilutions of trypsin. These tubes are placed in a bath at 50° C. for 15 or 30 minutes and then examined for the presence of clot. By noting the first tube of the series which shows absence of clotting, it is a simple matter to calculate the amount of the solution of trypsin necessary to neutralise the antitryptic power of a known volume of serum. This method has been fully described by Wright and Colebrook (1921).

(2) When sterile serum is not available, or in making large quantities, serum is obtained from blood procured from the slaughter-house, and to this is

added sufficient of any of the commercial glycerinated solutions of trypsin which are sold in bulk. Messrs. Allen & Hanburys, for instance, supply a suitable preparation under the name of *Liq. trypsinæ co.* Of this product 5 to 8 c.c. should be mixed with every 100 c.c. of horse-serum, as the trypsin content is not so high as that sent out in ampoules. The resulting mixture is then filtered through a porcelain candle of medium porosity and distributed with strict aseptic precautions into sterile vessels of suitable capacity.

Having obtained the reagents, the medium is prepared as follows: The agar is melted, and to every 100 c.c., 4 c.c. of the 1 per cent. potassium tellurite solution is added; this mixture is then cooled to between 65° and 60° C., and 15 c.c. of the trypsinised serum poured in. After thorough mixing, the medium is distributed into sterile tubes with precautions against contamination similar to those employed in making blood or serum agar, and at once sloped. The medium keeps well. The above proportion of trypsinised serum gives the most constant results, but less—for instance, 10 c.c. to every 100 c.c. of agar employed—is sufficient for all practical purposes.

THE APPEARANCE OF THE GROWTH RESULTING FROM IMPLANTATIONS FROM THROAT SWABS.

As the medium is perfectly transparent, and as colonies of the *B. diphtheriæ* and some organisms of the same group can be recognised with considerable certainty by inspection, it is advantageous to implant the surface somewhat sparsely so that individual colonies may have space to develop, and may be easily picked off for isolation or microscopical examination. A convenient practise is to draw the charged swab up the centre of the agar surface and then to distribute the material deposited over the whole surface by close strokes of a platinum loop, made at right angles to the track of the swab. After 18 to 24 hours' incubation, if *B. diphtheriæ* or certain other organisms of this group be present, colonies having the following characters will be apparent: in size they vary from about 0·75 to 1 mm. or more in diameter; in shape they resemble a somewhat flattened hemisphere with regular outline; in structure they are remarkably granular—a feature best made out with the aid of a hand lens; in colour, which is the most striking character, the centre is a dusky grey-black, while the peripheral portions are a somewhat opalescent greyish white, the whiteness being accentuated by the granular structure.

Colonies of Hofmann's bacillus have almost identical characters; the blackish colour of the centre is, however, usually less intense. *B. xerosis* and some other members of the diphtheroid group give colonies which are similar in every way to those of *B. diphtheriæ*.

Colonies of streptococci, which are usually the most abundant associated organisms, are very small, flat, and so translucent as to be visible only on close inspection. They have no trace of black colour at this stage of development. Only one organism has been experienced which at this stage of growth gives a colony in any way comparing in size and appearance with those of *B. diphtheriæ*. This is a staphylococcus which occurs in a small percentage of throat swabs. The colonies, however, differ from those of the diphtheroid group, mentioned above, in that the black colour is much more intense and more diffused throughout the colony. The granular structure, which is such a marked feature of colonies

of *B. diphtheriæ*, is replaced by a homogeneous smoothness—in fact, the colony resembles very closely a spot of wet black paint.

After incubation for 48 hours, the colonies of *B. diphtheriæ* have increased in size so that they may measure 2 or more millimetres in diameter. The black colour is much accentuated, and has spread throughout the whole colony. The central portion is usually somewhat thickened, the edges are smooth and regular; the general appearance at this and later stages of incubation may be compared to a flattened drop of partially dried black paint.

Colonies of streptococci have almost the same appearance as after 24 hours' incubation and usually remain inconspicuous. When examined carefully, however, a minute black spot may be made out in the centre of the colony, and this tends to become more marked after prolonged incubation.

From this description it is apparent that *B. diphtheriæ* and some other members of this group give colonies, after 24 hours' incubation, of a distinctive type, the principal features being their large size, granular structure, grey-black centre, and greyish-white peripheral portion. After 48 hours' incubation the colonies resemble a flattened drop of partially dried black paint. If no such colonies can be seen after 48 hours, the culture may be safely discarded as a negative result as regards the presence of *B. diphtheriæ*. Much greater caution is necessary in deciding by inspection that *B. diphtheriæ* is absent after 20 to 24 hours, as, unless the colonies are well separated, the distinctive characters may not have developed, and it is always advisable to make a microscopical preparation from any portion of the surface on which the growth is abundant.

When the colonies of staphylococcus are present, although with but little practice they can be distinguished with moderate certainty from those of the diphtheria group, microscopical examination is always necessary.

If isolation is desired, a convenient method is to draw out the end of a capillary pipette to a hair-fine tube in the peep flame of a Bunsen burner. This is then broken so that a portion of the hair-fine tube about 1 cm. long is left at the end. The suspicious colony is then touched with the point of the hair-fine tube. The pipette is then withdrawn and a single streak made up the centre of a fresh tube of medium. This deposits on the surface sufficient organisms to give an abundant culture, and separate colonies are assured by distributing these by close strokes of a platinum loop made at right angles to the central streak. The inside of the hair-fine tube will, on examination, be found to contain amply sufficient organisms to make a microscopical preparation, a plug of organisms, often half to one millimetre in length, having been drawn up by capillary attraction into the lumen. These can be easily washed out by placing a small drop of water on a slide, which is then sucked into and blown out of the capillary tube.

The microscopical appearance of *B. diphtheriæ* grown on this medium differs only from those grown on Loeffler's medium in that the metachromatic granules are not quite so abundant; they are, however, sufficiently numerous to make the use of the diagnostic stains based upon their presence perfectly satisfactory.

Virulence of emulsions of *B. diphtheriæ* was, in the case of nineteen strains investigated, similar in every way to that of emulsions made from cultures grown on Loeffler's medium.

RESULTS OBTAINED FROM THIS MEDIUM IN COMPARISON WITH THOSE FROM LOEFFLER'S MEDIUM.

In order to obtain a reliable result, the following technique was adopted:

Swabs were obtained from a number of cases of diphtheria admitted to the North-Western Fever Hospital, through the agency of Dr. Mair, to whom I now wish to convey my thanks. These cases were in all stages of the disease, and many of them had received antitoxin. On receipt of the swab the first process was to moisten it thoroughly in a tube of sterile broth; the second was to draw the swab up the centre of a tube of Loeffler's coagulated serum, rolling the swab during this process so that all aspects of the cotton-wool were brought into contact with the surface of the medium; the third was to carry out a similar implantation on to a tube of trypsinised serum agar; the fourth was to implant a tube of broth containing similar percentages of trypsinised serum and potassium tellurite solution. This last method was latterly discarded as unnecessary, as although organisms microscopically resembling *B. diphtheriæ* developed in all the broth tubes planted from swabs which gave colonies of this bacillus, such organisms were never found in the broth tubes when no colonies were seen on the solid medium.

From the results given in the table it will be seen that swabs from 29 cases were examined. *B. diphtheriæ* was found and isolated from the primary cultures from 19 of these by means of the trypsinised serum tellurite medium, while from Loeffler's medium *B. diphtheriæ* was microscopically recognised on 16 occasions, but isolation from primary cultures almost always failed. In no case was *B. diphtheriæ* found in the cultures on Loeffler's medium when absent from those on the trypsinised serum agar.

Medium employed.	No. of swabs examined.	No. of positive results.	Percentage of positive results.	No. of negative results.	Percentage of negative results.
Loeffler's medium .	29	16	55·2	13	44·8
Trypsinised serum-tellurite-agar .	29	19	65·5	10	34·5

All the cultures were virulent for guinea-pigs. It was especially in those swabs from which only a few colonies of *B. diphtheriæ* were obtained on the trypsinised serum tellurite agar that the employment of Loeffler's medium gave a negative result. Before deciding that a culture was negative it was always examined after 24 and 48 hours' incubation.

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NON-SPECIFIC DESENSITISATION.

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Received for publication August 18th, 1922.

THE phenomenon of non-specific desensitisation in anaphylactic animals has been studied by a number of observers, who have injected widely dissimilar substances to produce it. Thus Biedl and Kraus (1909) employed peptone, Kopaczewski and Vohran (1919) sodium oleate, Friedberger and Hartoch (1909) concentrated saline solutions, while Pfeiffer and Mita (1910) and later Weil (1914) used foreign sera. In a recent paper Karsner and Ecker (1922) have briefly reviewed the literature of this subject and have studied the desensitising effect of various foreign sera on the actively sensitive guinea-pig.

In the course of some experiments on the "anaphylatoxins," Dale and Kellaway (1922) found that normal guinea-pig serum, as well as that treated with starch, agar, etc., when administered intravenously could protect sensitive guinea-pigs from an otherwise fatal dose of antigen injected some time later. It was evident from their experiments that the time which elapsed after the desensitising injection was an important factor in determining the degree of protection conferred. In the experiments to be recorded here we have attempted to analyse this last type of non-specific desensitisation. Incidentally we have produced some further evidence in favour of the cellular view of anaphylaxis.

THE PROTECTION OF ACTIVELY SENSITIVE GUINEA-PIGS BY INTRAVENOUS INJECTION OF NORMAL GUINEA-PIG SERUM.

Our first concern was to ascertain the extent and duration of the protection afforded to the whole animal. A series of guinea-pigs were injected subcutaneously with 2 mgrm. of crystalline horse serum albumin, which was prepared for us by Dr. H. W. Dudley. Twenty-six days later the lethal dose of antigen was determined for the series as follows:

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Result.
350 grm.	0·2 mgrm.	Moderate symptoms; recovery in 6 minutes.
310 ,,	0·5 ,,	† in 4½ minutes.
260 ,,	0·5 ,,	† in 3½ ,,
280 ,,	0·5 ,,	† in 4½ ,,

The M.L.D. is 0·5 mgrm.

The fresh serum of four normal guinea-pigs was pooled and centrifugated, and 3 c.c. was injected intravenously into each of six remaining anaphylactic guinea-pigs of the above series. At different time intervals after this injection the sensitiveness of these animals was tested by the injection of a dose of antigen. The following results were obtained:

* Foulerton Student of the Royal Society.

Weight of guinea-pig.	Time interval after injection of serum.	Dose of antigen (horse serum albumin).	Result.
340 grm.	1 hour	1·0 mgrm.	No symptoms.
295 ,,	1 ,,	2·0 ,,	† in 11½ minutes.
340 ,,	2 hours	2·0 ,,	† in 4½ minutes.
340 ,,	3½ ,,	1·0 ,,	No symptoms.
320 ,,	22 ,,	0·5 ,,	Slight symptoms; recovery.
280 ,,	22 ,,	1·0 ,,	† in 8¾ minutes.

The protection afforded by the injection is not absolute. In this series it is maximal one hour after the injection, when twice the M.L.D. causes no symptoms, and four times the M.L.D. results in a somewhat delayed acute death. Two hours after the injection the protection is somewhat less, and three and a half hours after twice the M.L.D. still fails to produce symptoms. On the day following the injection the protection is greatly diminished, although the animal has not yet completely recovered its original sensitiveness. We now endeavoured to define the part played in the anaphylactic animal by the humeral and cellular defences in causing this temporary loss of sensitiveness. Dale and Kellaway regarded the protection afforded to the sensitive animal by serum containing "anaphylatoxin" and by normal serum as due to a "disturbance of the delicate balance of distribution of antibody between tissue cells and circulating fluid, on the maintenance of which the anaphylactic condition depends." We therefore first attempted to ascertain how the injection of normal guinea-pig serum affected the sensitiveness of the isolated plain muscle.

THE SENSITIVENESS OF PLAIN MUSCLE IN ACTIVELY SENSITIVE GUINEA-PIGS AFTER THE INJECTION OF SERUM.

Three series of young virgin female guinea-pigs were actively sensitised by the subcutaneous injection of 1 mgrm. of horse serum albumin. Uniformity of sensitiveness within each series was obtained by avoiding overcrowding and providing a plentiful supply of food during the acquisition of sensitiveness. To test the sensitiveness of the plain muscle the apparatus of Dale and Laidlaw (1912) was used. The two uterine horns, after thorough perfusion by the method of Dale (1913), were separately tested in a bath of oxygenated Ringer of 120 c.c. capacity.

Series 1: *Guinea-pigs* 38 *to* 41 *days after the Sensitising Injection.*

Of 11 animals of this series, 4 served as controls. As a further control 2 were tested 27 and 30 hours respectively after specific desensitisation by a second injection of 1 mgrm. of the antigen subcutaneously. The remaining 5 were treated with serum as follows: Four large normal guinea-pigs were bled out and the serum was pooled and centrifugated. Of this pooled serum 5 c.c. was injected intravenously into each of the anaphylactic guinea-pigs. Three of these were tested 4, 24 and 27 hours respectively after the injection of the fresh serum, and two were tested 5 and 6 hours after the injection of the same serum when it was 24 hours old. The following results were obtained in the untreated controls:

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
285 grm.	0·02 mgrm.	Small, delayed.
	0·1 „	Maximal.
290 „	0·04 mgrm.	Maximal.
	0·1 „	„
240 „	0·04 mgrm.	*Nil.*
	0·1 „	Maximal.
290 „	0·04 mgrm.	Large but poorly sustained.
	0·1 „	Maximal.

The sensitiveness of the plain muscle of these control animals was fairly uniform. In all cases a maximal reaction was obtained with a concentration of antigen of 1 in 1,200,000. The individual variation in sensitiveness was shown by the reaction of the second horn, which to a concentration of 1 in 3,000,000 was maximal in one case, modified in another and *nil* in a third. The second horn of the remaining guinea-pig gave a small delayed response to a concentration of 1 in 6,000,000.

In contrast to these normal reactions are those of the three animals treated 4, 5 and 6 hours previously by the intravenous injection of serum.

Weight of guinea-pig.	Time interval.	Dose of antigen.	Reaction of plain muscle.
250 grm.	4 hours	0·1 mgrm.	*Nil.*
		1·0 „	Barely perceptible.
250 grm.	5 hours	0·1 mgrm.	Moderate.
		1·0 „	Maximal.
200 grm.	6 hours	0·04 mgrm.	Moderate.
		0·1 „	Nearly maximal.

The guinea-pig which was allowed to survive 4 hours was injected with fresh serum, whereas the other two animals received the same serum when it was 24 hours old. The plain muscle of the first showed a marked loss of sensitiveness, reacting hardly at all to a concentration of 1 in 120,000. The uterus of the guinea-pig which was allowed to survive 5 hours after the serum injection showed only a slight loss of sensitiveness. Six hours after the injection of 24-hour-old serum there was no appreciable loss of sensitiveness.

The responses of the plain muscle of guinea-pigs 24 and 27 hours after the injection of fresh serum were as follows:

Weight of guinea-pig.	Time interval.	Dose of antigen.	Reaction of plain muscle.
270 grm.	24 hours	0·04 mgrm.	Delayed, modified.
		0·1 „	Maximal.
230 grm.	27 hours	0·1 mgrm.	Maximal.

The plain muscle at this period after the injection shows no evidence of diminished sensitiveness. In contrast to these results was the specific desensitising effect of the subcutaneous injection of 1 mgrm. of horse serum albumin into animals tested 24 and 27 hours afterwards. Their plain muscle failed to give the slightest trace of reaction to a concentration of 1 in 12,000.

This preliminary series yielded no information concerning the changes in

the sensitiveness of plain muscle occurring earlier than 4 hours after the injection of serum. In the next series we were concerned particularly with these early changes.

Series 2: *Guinea-pigs* 63 *to* 64 *days after the Sensitising Injection.*

Of 7 animals in this series 2 served as controls, and the remaining 5 were tested $\frac{1}{4}$, $\frac{1}{2}$, 1, 2 and $3\frac{3}{4}$ hours respectively after the intravenous injection of 5 c.c. of pooled normal guinea-pig serum 24 hours old.

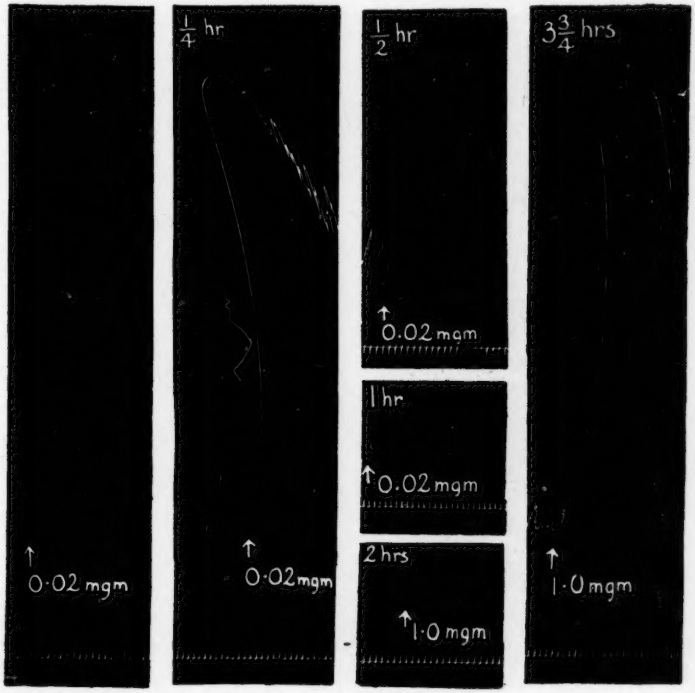

FIG. 1.—Plain muscle reactions of actively sensitive guinea-pigs. Antigen, horse serum albumin. Tracing on left from untreated control; others from animals $\frac{1}{4}$, $\frac{1}{2}$, 1, 2 and $3\frac{3}{4}$ hours after desensitising injection. Time in half minutes.

The control animals gave the following results:

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
330 grm.	0·02 mgrm.	Maximal.
	0·005 „	„
250 „	0·01 „	Maximal.

The plain muscle was highly sensitive, reacting to 1 in 12,000,000 of the antigen. The limits of sensitiveness were not determined.

The following results were obtained after the injection of serum:

Weight of guinea-pig.	Time interval.	Dose of antigen (horse serum albumin.)	Reaction of plain muscle.
290 grm.	¼ hour	0·1 mgrm.	Maximal.
		0·02 „	„
315 „	½ hour	0·1 mgrm.	Delayed, modified.
		0·02 „	„
290 „	1 hour	0·1 mgrm.	Small
		0·02 „	Trivial, delayed.
350 „	2 hours	0·1 mgrm.	*Nil.*
		1·0 „	„
300 „	3¾ hours	0·1 mgrm.	Maximal.
		1·0 „	„

The early changes in the sensitiveness of the plain muscle after the injection of serum are well illustrated by this series (see also Fig. 1). In the first ¼ hour after the injection there is no significant change in the reaction of the uterus. Half an hour after the serum injection there is a small change, the plain muscle giving only a modified response to a concentration of antigen of 1 in 1,200,000. One hour after the injection a further small loss of sensitiveness has occurred. Two hours after there is no trace of reaction to a concentration of 1 in 120,000. Three and three-quarter hours after the injection the plain muscle reacts almost maximally to a concentration of 1 in 1,200,000—that is to say, it has regained much of its original sensitiveness.

Series 3: *Guinea-pigs* 21 *to* 24 *days after the Sensitising Injection of Horse Serum Albumin.*

Here fresh-pooled guinea-pig's serum was used for desensitisation instead of serum 24 hours old as in the last series. Of seven sensitive guinea-pigs three served as controls, and the plain muscle of the remaining four animals was tested ¼ hour, ½ hour, 2 hours and 4 hours after the intravenous injection of 3 c.c. of fresh serum.

The plain muscle of two of the controls was tested, with the following results:

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
210 grm.	0·1 mgrm.	Maximal.
	0·02 „	„
215 „	0·1 mgrm.	Maximal.
	0·02 „	Small, delayed.

The plain muscle was uniformly sensitive to a concentration of antigen of 1 in 1,200,000. Variations appeared in the reaction of the second horn to smaller doses.

The following results were obtained in the guinea-pigs injected with serum (see also Fig. 2):

Weight of guinea-pig.	Time interval.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
190 grm.	¼ hour	0·1 mgrm.	Small.
		1·0 „	Slow but maximal.

Weight of guinea-pig.	Time interval.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
205 grm.	½ hour	0·1 mgrm.	*Nil.*
		1·0 „	Small.
205 „	2 hours	1·0 mgrm.	Small.
		10·0 „	Moderate.
205 „	4 „	0·1 mgrm.	Nearly maximal.
		0·02 „	Small.

This series differs from the last in that some change in the sensitiveness of the plain muscle was detected as early as ¼ hour after the injection of serum. The use of fresh instead of 24-hour-old serum may account for this difference. After ½ hour the plain muscle was very greatly diminished in sensitiveness, giving only a small reaction to a concentration of 1 in 120,000. Two hours after the injection the reaction of the plain muscle was about the same in this concentration, and a moderate response was obtained from the second horn to a concentration of 1 in 12,000. After 4 hours the sensitiveness of the plain muscle had almost returned to its original value.

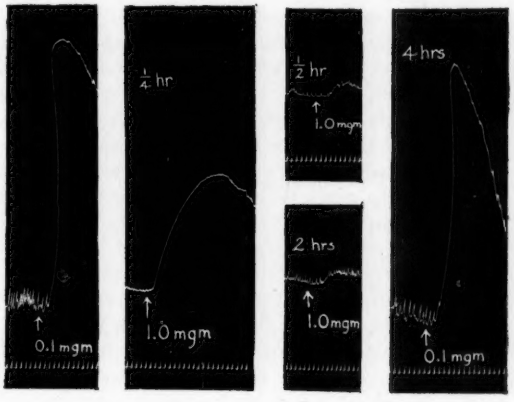

FIG. 2.—Plain muscle reactions of actively sensitive guinea-pigs. Antigen, horse serum albumin. Tracing on left from untreated control; others from animals ¼, ½, 2 and 4 hours after desensitising injection. Time in half minutes.

We may now summarise the changes in sensitiveness of the plain muscle which follow the intravenous injection of normal guinea-pig serum and compare them with the changes in sensitiveness of the whole animal which we have already described. Little change in the sensitiveness of the plain muscle takes place in the first ½ hour after the injection, unless fresh serum is used, in which case diminution of sensitiveness appears to occur earlier. After 1 hour desensitisation is well marked, and at the end of 2 hours it appears to have reached its maximum, though it is not complete. Four hours after the injection sensitiveness has returned to somewhere near its original level. Finally, on the following day little or no diminution of sensitiveness can be detected.

The changes in the sensitiveness of the whole animal correspond closely to those of the plain muscle. There is one apparent divergence: 24 hours after the injection the animal still has some protection against the lethal dose of antigen, while at this time the sensitiveness of the plain muscle appears to be completely restored within the limits of individual variation. It is doubtful whether the small change in the sensitiveness of the plain muscle which would correspond to the degree of protection observed in the whole animal at this period could be detected by the methods which we have employed.

There are several possible explanations of the changes in the sensitiveness of the plain muscle :

(1) The muscle cells may throw off fixed antibody into the circulation.

(2) A substance may be formed which inhibits the normal anaphylactic reaction of the animal.

(3) The fixed antibody may be destroyed *in situ* and rapidly re-formed.

(4) A temporary physical change may occur in the muscle cells which masks the presence of fixed antibody—(*a*) by preventing the response of the plain muscle to the interaction of antibody and antigen, or (*b*) by preventing this interaction from occurring.

(1) Does the injection of serum cause the plain muscle to throw off antibody into the circulating fluid ?

In order to answer this question we investigated a series of guinea-pigs (Series 2) 9 weeks after the sensitising injection. At this period of active sensitiveness, as Weil (1913) [1 and 2] showed, no antibody is demonstrable in the serum, though the plain muscle is highly sensitive. The plain muscle reactions of this series have already been described. The whole of the serum of each of the animals in the series was injected intraperitoneally into a normal virgin guinea-pig, and 48 hours later the sensitiveness of the plain muscle of this guinea-pig was tested. It was found that the serum of the untreated anaphylactic animals failed to confer any passive sensitiveness on the normal animal, the plain muscle of the latter showing no reaction to a concentration of antigen of 1 in 12,000. The serum of anaphylactic animals $\frac{1}{4}$ hour, $\frac{1}{2}$ hour, 1 hour, 2 hours and $3\frac{3}{4}$ hours after the injection of serum likewise failed to produce any trace of passive sensitiveness in their corresponding normal guinea-pigs. These results seem to negative the possibility under consideration, though it may be argued that the method employed is not sensitive enough for the detection of the very small changes in circulating antibody involved. In any case the rapid return of sensitiveness remains to be explained, either by (*a*) the re-formation of antibody—a possibility which is considered later, or (*b*) the re-fixation of circulating antibody—a supposition which is rendered improbable by the known facts concerning the time required for fixation of homologous antibody in passive sensitisation.

(2) Does the injection of serum provoke the formation of a substance which inhibits the normal anaphylactic reaction of the plain muscle ?

If such a substance is formed it must be present within the plain muscle cells themselves, since the blood has been thoroughly washed away from the uterus by perfusion. There is no evidence in our experiments that prolonged perfusion followed by immersion for some hours in repeated changes of oxygenated Ringer causes any alteration in the degree of desensitisation of the plain muscle. If such an inhibitory substance were responsible it is likely that this treatment would cause some restoration of sensitivenes *in vitro*, and in the absence of such evidence it seems unwise to assume the formation of such an inhibitory substance.

(3) Is the antibody fixed in the plain muscle-cells destroyed and rapidly re-formed ?

The experiments which we have so far described might be explained on this hypothesis. It seemed a little dubious whether, if destruction of antibody

took place in this way, re-formation could occur so rapidly that, with maximal desensitisation at the end of the second hour after the injection of serum, the sensitiveness of the plain muscle could be almost completely restored in the course of the next two hours by the re-formation of antibody. We were able to put this matter to the test of experiment.

Though it is conceivable that such rapid re-formation of *homologous* antibody might occur, any re-formation in the case of *heterologous* antibody is manifestly impossible. We therefore investigated the phenomenon of non-specific desensitisation in guinea-pigs passively sensitised with specific antibody produced in animals of another species.

A rabbit weighing 2460 grm. was immunised by a series of seven injections of from 2 c.c. to 5 c.c. of horse serum at intervals of 7 days. Six days after the last injection its serum diluted five times gave a good precipitate with a 1 in 500 dilution of horse serum. It was now bled out and 2 c.c. of the clear centrifugated serum was injected intraperitoneally into each of a series of guinea-pigs. Forty-four to 48 hours later these guinea-pigs were tested as follows:

Weight of guinea-pig.	Dose of horse serum.	Result.
220 grm.	0·1 c.c.	† in 4 minutes.
300 ,,	0·02 ,,	† in 6 ,,
240 ,,	0·02 ,,	† in 6 ,,
240 ,,	0·005 ,,	Slight symptoms; recovery.

Four other animals of this series were injected intravenously with 3 c.c. of fresh normal guinea-pig serum and tested at various periods after the injection, with the following results:

Weight of guinea-pig.	Time interval.	Dose of horse serum.	Result.
250 grm.	1 hour	0·1 c.c.	† in 5¼ minutes.
240 ,,	1¼ hours	0·02 ,,	Very slight symptoms.
300 ,,	2¼ ,,	0·02 ,,	Severe symptoms; recovery in 8 minutes.
300 ,,	4 ,,	0·02 ,,	† in 4 minutes.

These animals show no very great loss of sensitiveness, since five times the M.L.D. kills quickly 1 hour after the injection of serum. There is some protection against 1 M.L.D. until 2¼ hours after the injection. This loss of sensitiveness has disappeared by the fourth hour. The protecting effect is similar to that observed in actively sensitive animals, appearing and disappearing at corresponding periods.

The plain muscle reactions of two guinea-pigs of this series, investigated at the same period after the sensitising injection, one of which had received an intravenous injection of 3 c.c. of fresh normal guinea-pig serum, were as follows:

Weight of guinea-pig.	Treatment.	Dose of horse serum.	Reaction of plain muscle.
240 grm.	P.S. 64 hours	0·02 c.c. 0·01 ,,	Moderate. ,,
240 ,,	P.S. 66 hours serum injected 22 hours	0·01 c.c. 0·1 ,,	Small. Maximal.

There is but little difference in the reactions; much of the loss of sensitiveness due to the serum injection must therefore have been repaired at this time. While it is just possible that such repair may have been achieved by the formation of homologous antibody, it is much more probable that the return of sensitiveness does not take place by the re-formation of antibody at all. If this be so, it is clear that loss of sensitiveness can be due neither to destruction of antibody nor to its being thrown off into the circulation.

(4) Does a temporary physical change take place in the plain muscle as the result of the injection of serum which masks the presence of fixed antibody?

Failing the discharge of antibody from the cell, its destruction, or the formation of some inhibitory substance, we are left with the hypothesis that some physical change in the muscle-cell is responsible for its loss of sensitiveness. We are not concerned here with any gross alterations in the tonicity of the circulating fluids which would disturb the anaphylactic response of the plain muscle, as has been shown by Dale (1913)[2]. Sensitiveness is not restored to the plain muscle of non-specifically desensitised animals by perfusion with properly balanced saline solutions. Moreover we have found that at the period of maximal desensitisation the isolated plain muscle exhibits its normal rhythm, and is capable of responding with a normal contraction to adequate stimuli, such as small doses of histamine.

The nature of this change, which must be reversible and may be concerned with the degree of aggregation of the cell colloids, and its mode of action, whether by preventing contraction in response to the union of antibody and antigen, by preventing this union, or by diminishing its reaction velocity, must be left for further investigation.

THE CHANGES IN THE DEMONSTRABLE ANTIBODY IN THE CIRCULATING FLUIDS OF THE BODY WHICH RESULT FROM THE INJECTION OF SERUM.

The methods which we have applied to the investigation of changes in circulating antibody are less sensitive than those available for the study of fixed antibody. The guinea-pig is not a suitable animal in which to study changes in the titre of precipitin in its serum, and most of our evidence is concerned with somewhat rough attempts to gauge the amount of antibody present by its capacity to transfer sensitiveness to normal guinea-pigs.

Series 3: *Experiments with Guinea-pigs* 21 *to* 24 *days sensitive.*

The changes in the sensitiveness of plain muscle of these animals have already been recorded. The method of investigating changes in the circulating antibody was to inject the whole of the serum from each animal in the series into a normal guinea-pig and to test its sensitiveness 48 hours later by the intravenous injection of the antigen. The serum of three untreated controls in this series, injected intraperitoneally into normal guinea-pigs, caused the appearance of passive sensitiveness in these latter animals:

Weight of guinea-pig.	Amount of serum injected I.P.	Dose of antigen (horse serum albumin).	Result.
230 grm.	3 c.c.	2·0 mgrm.	Moderate symptoms.
210 ,,	4 ,,	5·0 ,,	† in 4¾ minutes.
290 ,,	4 ,,	5·0 ,,	† in 7½ ,,

The serum of the sensitive guinea-pigs into which 3 c.c. of fresh normal guinea-pig serum had been injected intravenously had lost, at least in part, this capacity for conferring passive sensitiveness. Three such guinea-pigs, ¼ hour, ½ hour and 4 hours respectively after the injection of serum, yielded 4½ c.c., 5 c.c. and 3½ c.c. of serum respectively. The animals injected intraperitoneally with these sera presented no trace of symptoms when 10 mgrm. of horse serum albumin was injected intravenously 48 hours later.

Fifteen minutes after the injection of normal guinea-pig serum there is a noteworthy diminution in the capacity of the serum of the anaphylactic animal to confer passive sensitiveness, which persists until 4 hours after the injection. Dilution of the circulating fluid in the sensitive guinea-pig by the injection of serum cannot be held responsible for this phenomenon, because the whole of the serum obtained from each sensitive animal was injected into the corresponding normal guinea-pig. It is possible that the presence of the serum injected to produce desensitisation, and consequently admixed with the serum which is injected intraperitoneally to produce passive sensitiveness, may prevent such transfer of sensitiveness even if effective antibody is present in sufficient titre. That this is not the explanation of the failure to transfer sensitiveness with the serum of such injected animals can be inferred from experiments recorded in the last section of this paper.

Series 1: *Experiments with Guinea-pigs* 38–41 *days sensitive.*

The serum of the four untreated controls of this series was pooled, and a concentrated globulin solution (A) was prepared from it in the manner described by Dale and Kellaway (1922). A similar solution (B) was obtained from the pooled serum of the three guinea-pigs which were allowed to survive 4, 5 and 6 hours after the injection of serum. The sera of the two guinea-pigs allowed to survive 24 and 27 hours after the injection yielded the globulin solution (C). These solutions were made up with saline to volumes corresponding to the number of animals from which they were obtained (A) to 8 c.c., (B) to 6 c.c., and (C) to 4 c.c.

Solutions (A) and (B) were centrifugated to free them from suspended particles and their precipitin titres were compared. (A) diluted 10 times gave a faint precipitate against 1 in 5000 of the antigen; (B) treated similarly gave no trace of precipitate in any dilution. We cannot lay any stress on these results because the amount of precipitate with (A) was very small.

The globulin solutions were now tested for their capacity to transfer sensitiveness to normal virgin female guinea-pigs. The plain muscle of these animals was tested 48 hours later, with the following results:

Weight of guinea-pig.	Solution injected I.P.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
200 grm.	2 c.c. (A)	1·0 mgrm.	Small, delayed.
		5·0 ,,	Maximal.
200 ,,	2 ,, (B)	10·0 mgrm.	*Nil.*
250 ,,	2 ,, (B)	10·0 ,,	,,
200 ,,	2 ,, (C)	10·0 ,,	,,
200 ,,	2 ,, (C)	10·0 ,,	,,

The method used here of testing the plain muscle of female guinea-pigs for passive sensitiveness permits of greater accuracy with a limited number of animals than the method of testing for sensitiveness in the whole animal. From the above experiment it would seem that the capacity of the serum of actively sensitised guinea-pigs for transferring sensitiveness is greatly diminished by the injection of serum, and remains so till the day following the injection.

Series 4: *Immune Guinea-pigs.*

The later changes in the circulating antibody were studied in a group of five immune female guinea-pigs. These animals had received 6 subcutaneous injections of 0·5 c.c. of horse serum at intervals of 7 days, and the experiments were carried out 24 days after the last injection. Two of the animals served as controls, and the remaining three were injected intravenously with 3 c.c. of fresh pooled guinea-pig serum. The controls were bled out and the plain muscle was perfused and tested. It reacted maximally to a dose of 0·1 c.c. of horse serum and gave a good reaction to 0·01 c.c. The guinea-pigs which had been injected with serum were bled out, and their plain muscle was tested 20, 66 and 88 hours respectively after the injection. Their perfused plain muscle gave similar reactions to those yielded by the uteri of untreated guinea-pigs of this series. There was therefore no great difference in the degree of immunisation of the individual members of the series.

Four c.c. of serum from each of these immune guinea-pigs was injected intraperitoneally into a normal female guinea-pig, and after the lapse of 48 hours the passive sensitiveness of the plain muscle of this corresponding series of animals was tested. The following results were obtained:

Weight of guinea-pig passively sensitised.	Serum injected intraperitoneally.	Dose of horse serum.	Reaction of plain muscle.
450 grm.	Untreated immune	0·02 c.c.	Large, poorly sustained.
		0·1 „	Nearly maximal.
350 „	„	0·02 c.c.	Nearly maximal.
250 „	Immune, 20 hours after serum injection	0·1 c.c.	Barely perceptible.
		1·0 „	Small.
300 „	Immune, 66 hours after serum injection	0·1 c.c.	Large, poorly sustained.
		1·0 „	Nearly maximal.
400 „	Immune, 88 hours after serum injection	0·1 c.c.	Large, poorly sustained.
		1·0 „	Maximal.

The serum from the immune guinea-pig 20 hours after the injection of serum showed a well-marked loss of the capacity to transfer sensitiveness, but the serum from animals killed 66 to 88 hours after the desensitising injection was nearly as effective in producing passive sensitiveness as that from the untreated immune animals.

Summary of the changes in demonstrable circulating antibody after the injection of serum.—A marked decrease in the demonstrable circulating antibody occurs as early as 15 minutes after the injection of normal guinea-pig serum. There is no evidence of its return to normal until some time between

the 24th and 66th hour after the injection; even after 88 hours it has not been completely restored. We have not yet investigated the cause of these changes. Despite the difference in time relations, it is not likely to be essentially different from that which operates in the case of the plain muscle.

THE DIFFERENCE IN TIME RELATIONS BETWEEN THE CHANGES IN DEMONSTRABLE CIRCULATING ANTIBODY AND CHANGES IN PLAIN MUSCLE SENSITIVENESS.

In the diagram (Fig. 3) we have represented very roughly the difference in the time relations of these two sets of changes. The titre of antibody is plotted against the time intervals following the injection of serum. The interrupted curve represents the changes in circulating antibody and the continuous one those occurring in fixed antibody.

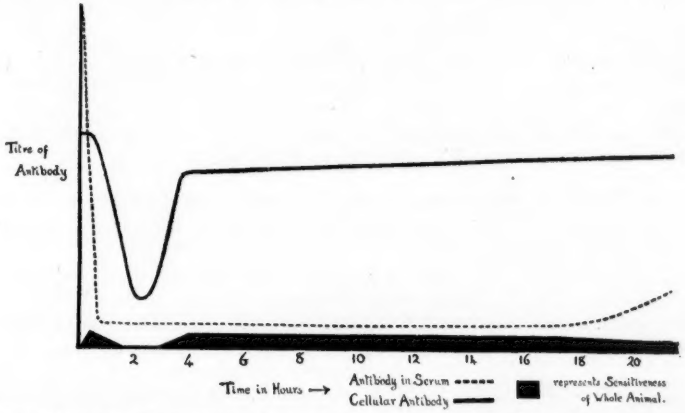

FIG. 3.—Schematic representation of circulating and fixed antibody in an immune guinea-pig. Continuous line = fixed; interrupted line = circulating antibody.

According to Dale and to Weil the anaphylactic reaction is dependent upon the union of antigen with fixed antibody, and the essential difference between immune and sensitive animals is that in the former sufficient circulating antibody is present to unite with the injected antigen and prevent its access to the sensitive cell. The diagram has been drawn so as to represent the effect of the injection of serum in a relatively immune guinea-pig. The titre of circulating antibody is represented as being at a higher level than that of fixed antibody before the injection. If the cellular view of anaphylaxis is the correct one, and if we assume that the antibody in the circulating fluid rapidly loses, as the result of the injection, not only its capacity for the transference of passive sensitiveness, but also, as is suggested by our precipitin experiments, its capacity for union with antigen, it ought to be possible to demonstrate, in any animal with a high titre of circulating antibody, two periods of increased sensitiveness of the whole animal after the injection of serum. Since the changes in the circulating antibody commence earlier than the loss of sensitiveness of the plain muscle, there should be a short period of enhanced

sensitiveness soon after the injection. After the plain muscle has recovered its sensitiveness, *i.e.* after the fourth hour, there should be a second more prolonged period of increased sensitiveness. We have attempted to demonstrate the occurrence of this increased sensitiveness in animals with a high titre of circulating antibody.

Series 5 : *Guinea-pigs Actively Sensitised by a Series of Subcutaneous Injections.*

Eight guinea-pigs received a series of 5 subcutaneous injections of 1 mgrm. of horse serum albumin at intervals of 7 days. They were investigated 34 days after the last injection. Similarly treated animals at the same period after the last injection had been found to possess a moderately high titre of circulating antibody. We first determined the M.L.D. of antigen for the series :

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Result.
350 grm.	0·5 mgrm.	No symptoms.
350 ,,	0·5 ,,	,,
290 ,,	1·0 ,,	† in 3 minutes.

The M.L.D. is 1 mgrm.

The remaining 5 guinea-pigs were injected intravenously with 4 c.c. of fresh pooled serum. Two were tested half an hour after this injection and gave the following results :

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Result.
290 grm.	0·5 mgrm.	† in 6 minutes.
440 ,,	0·5 ,,	† ,, 5 ,,

There was thus a definite increase of sensitiveness following the injection of the serum.

The remaining three animals were tested between the 21st and 24th hour after the injection in order to demonstrate if possible the second period of enhanced sensitiveness :

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Result.
410 grm.	0·5 mgrm.	Very severe symptoms ; only just recovered.
320 ,,	0·5 ,,	Severe symptoms ; recovery.
500 ,,	0·5 ,,	,, ,, ,,

Here a definite increase in sensitiveness was present, though it was not as great as that shown in the early period after injection.

Series 6 : *Immune Guinea-pigs.*

This series of animals received six doses of 0·5 c.c. of horse serum subcutaneously at intervals of 7 days. They were tested 24 days after the last injection, when 4 c.c. of serum from one of them injected intraperitoneally into a normal female guinea-pig rendered its plain muscle sensitive to a dose

of 0·02 c.c. of horse-serum. The effect of a dose of 0·5 c.c. of horse-serum on the untreated animals of this series was first tested :

Weight of guinea-pig.	Dose of horse serum.	Result.
390 grm.	0·5 c.c.	Slight symptoms ; recovery.
500 ,,	0·5 ,,	,, ,, ,,
450 ,,	0·5 ,,	Severe symptoms ; recovery.

The remaining guinea-pigs of the series were injected with 2 c.c. of fresh normal guinea-pig serum. Three of these were tested by the intravenous injection of antigen half an hour after the administration of serum :

Weight of guinea-pig.	Dose of antigen (horse-serum).	Result.
380 grm.	0·25 c.c.	Slight symptoms ; recovery.
360 ,,	0·5 ,,	† in $3\frac{3}{4}$ minutes.
440 ,,	0·5 ,,	† in $5\frac{1}{4}$,,

The remaining guinea-pigs were tested 20 hours after the serum injection :

Weight of guinea-pig.	Dose of antigen (horse serum).	Result.
410 grm.	0·5 c.c.	† in 30 minutes.
310 ,,	0·5 ,,	† in $3\frac{1}{4}$,,
400 ,,	0·5 ,,	Severe symptoms.*

There seems to be no doubt concerning the genuineness of this phenomenon of increase of sensitiveness at two different periods after the injection of homologous serum. In this last experiment the increase was not so striking as in the previous one, since 0·5 c.c. of horse serum was probably not much less than the lethal dose of antigen for the untreated guinea-pigs. The small size of the dose of the normal guinea-pig serum in this last experiment may account for this difference in the two series.

In view of these results we must recognise that if circulating antibody is not destroyed after the injection of serum, it loses not only its power of transferring sensitiveness, but also its capacity for union with antigen. In other words, the only two characters possessed by antibody, by which we are enabled to recognise its presence in the circulating fluids of the body, are lost.

The experiments which we have described here are in accordance with the "cellular" theory of anaphylaxis, and are extremely difficult to interpret in terms of the rival theory which attributes the anaphylactic phenomenon to the formation of a hypothetical "anaphylatoxin."

CONCLUSIONS.

(1) The intravenous injection of normal guinea-pig serum into actively sensitive guinea-pigs causes a degree of protection against the specific antigen which is only of short duration.

(2) This loss and subsequent return of the sensitiveness of the anaphylactic animal is explained by parallel changes in the sensitiveness of the plain muscle.

* This animal had apparently recovered at the end of 10 minutes and was then killed. A similar recovery was shown by the guinea-pig which died with circulatory collapse at the end of half an hour.

(3) The changes in the sensitiveness of the plain muscle are probably due to physical changes occurring in the muscle cells.

(4) The demonstrable antibody of the serum is greatly diminished in amount very soon after the injection of serum, and is not restored till long after the sensitiveness of the plain muscle has returned to its original value.

(5) The injection of guinea-pig serum into guinea-pigs with a high titre of circulating antibody is followed by the appearance of enhanced sensitiveness of the whole animal during two periods—one shortly after the injection, and a second after the restoration of the sensitiveness of the plain muscle but before the return to normal of the demonstrable circulating antibody.

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ON THE OCCURRENCE OF A TOXIC SUBSTANCE IN THE BLOOD IN CASES OF BRONCHIAL ASTHMA, URTICARIA, EPILEPSY, AND MIGRAINE.

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Received for publication September 20th, 1922.

IT has become clear during the last few years that illnesses like asthma, hay fever, urticaria, etc., have, in most instances, to be considered as "allergic diseases," which means that these pathological conditions are due to hypersensitiveness to substances which are innocuous for normal people. These substances, causing attacks of asthma or hay fever or urticaria, may be pollen, or some animal protein, or some foodstuff or drug, or, as highly interesting experiments of Cooke (1922) have shown, an unknown substance occurring n "house" dust.

Without any doubt this conception holds good for most cases of asthma and allied diseases, and consequently it can be stated that in the greater

number of these cases the direct cause of the attack is known. Since the agents causing an attack in certain individuals are completely innocuous for normal persons, it must be surmised that asthmatics, sufferers from hay fever, urticaria and so forth possess a certain "disposition" to allergic reactions. Of the nature of this "disposition" or "allergic condition" little is known. An attempt has often been made to demonstrate that this condition is an anaphylactic one, but this view, as has been emphasised by Coca, Cooke and others, must be abandoned. The allergic condition may be related to anaphylaxis, but it is not a true anaphylactic phenomena, such as occurs in animal experiments.

Moreover, it is known that in allergic diseases hereditary factors play an important *rôle*. Cooke and van der Veer (1916) find among 504 cases of hay fever hereditary antecedents in 48·4 per cent.

de Kleyn (1912), in collaboration with one of the present writers, showed that most asthmatics present a disordered purine excretion, whilst Storm van Leeuwen and Varekamp (1921) drew attention to the fact that a great number of sufferers from asthma and hay fever give a considerable v. Pirquet tuberculin reaction, and are highly benefited by tuberculin treatment. In addition it is known that in allergic conditions there mostly is a hypersensitiveness to *several* agents; more than one animal protein, animal protein + pollen, animal protein + aspirin, and so forth. Such patients are apt to be of a nervous disposition, so that there is considered to exist a hyperexcitability of the sympathetic system.

All these things are of considerable interest, but they do not offer a satisfactory explanation of the mechanism of allergic disposition.

In view of this state of affairs the present authors felt justified in facing the problem from a different angle, and they adopted the following working hypothesis.

Apart from all other considerations stated above, the asthmatic condition is—or, generally speaking, allergic conditions are—due to a hyperexcitability of smooth musculature in the lung (or other organ). *This hyperexcitability is due to a substance present in the blood of these patients, a substance which has the property of stimulating smooth muscle.*

The testing of this hypothesis seemed simple at first. Blood of a number of asthmatics was taken, and allowed to clot. After 24 hours the serum was taken off and its action on isolated organs was tested. It has long been known that normal serum contains substances which stimulate smooth musculature; and recently one of the present authors and his co-workers (Storm van Leeuwen, 1921) showed that it also contains substances which are able to augment the action of certain drugs. This action of normal serum of course offered a difficulty in the testing of sera of pathological cases, but we hoped to be able to show that the serum of an asthmatic would be more effective in one or the other way than normal serum. However, we were disappointed in this respect, for although the serum of asthmatics was, as a rule, highly active, it was not more active than some normal human sera. It therefore became necessary to devise a method which would enable the stimulating and augmentor substances occurring in normal serum to be removed. Our attention was then drawn to a recent publication of Freund

(1921), who, with a different purpose, was faced with the same problem, viz. the elimination of the active substances of normal serum. It is conceived that these substances are not present in the uncoagulated blood, but only form during clotting. Experiments of Trendelenburg (1916), however, have shown that although freshly drawn blood is free from these substances, they appear, even if clotting is prevented by sodium oxalate, within half a minute after the blood has been shed. Starting from this fact, Freund worked out the following method: Blood is drawn from a large vein with a thick needle, and allowed to flow freely into a flask containing 96 per cent. alcohol. The alcohol is then evaporated off at low temperature, and the residue taken up in physiological saline. This solution if obtained from normal individuals does not contain stimulating substances.

It seemed to us that Freund's method might prove useful for our purposes and we performed the following experiments:

Blood was drawn, in the way described by Freund, from 23 men, viz. 10 normals, 9 asthmatics, 2 sufferers from intense urticaria, 1 epileptic and 1 case of migraine.

From every sample of blood an alcoholic extract was made and all the extracts were tested on the isolated gut of the cat. We chose this procedure because it has proved in our hands to be one of the best and most convenient methods for studying the action of drugs on smooth musculature. In all experiments the sensitiveness of the gut for pilocarpine was first tested, and only after a constant sensitiveness to this drug was established was the blood extract to be tested added. This enabled us (1) to calculate roughly the amount of stimulating substance present in a certain blood, comparing its effect with that of pilocarpine, and (2) to judge whether in a given extract substances might be present which show an augmentor effect on pilocarpine action.

Briefly stated the results of these experiments were as follow:

The extracts of all the normals showed no action at all on the gut, if doses corresponding to 4–12 c.c. of blood were used.

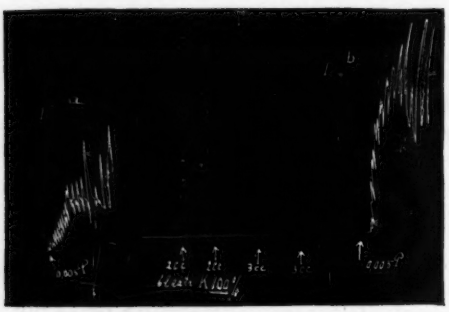

Fig. 1.—Extract of normal blood has no influence on isolated gut. *a*. 0·005 mgrm. pilocarpine hydrochloride added to 75 c.c. tyrode solution gives moderate contraction of gut. *b*. 10 c.c. blood extract corresponding to 10 c.c. blood from normal man gives no contraction; 0·005 mgrm. pilocarpine subsequently given produces strong contraction.

The extract of the blood of one asthmatic (very light case who had only attacks occasionally) was also negative. *In all the other cases of asthma and in the cases of urticaria and migraine the blood extract contained a substance which had a very definite action on the smooth muscle of the gut.* Roughly calculated 1 litre of blood contained a quantity of the unknown poison which had an action similar to that of 2–5 mgrm. of pilocarpine. The slight cases had less of this poison in the blood than the severe cases. In the one case of epilepsy which was studied only a small amount of the toxic substance was found. Figs. 1 to 3 give instances of the experiments described, whereas Tables I and II give the results of all the experiments made.

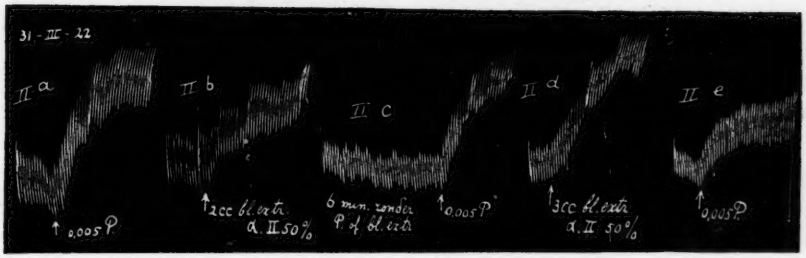

FIG. 2.—Influence of blood extract from severe case of asthma on isolated gut. *a*. 0·005 mgrm. pilocarpine gives moderate contraction. *b*. 2 c.c. blood extract corresponding to 1 c.c. blood gives contraction of gut. *c*. Action of 0·005 mgrm. pilocarpine. *d*. Action of 3 c.c. blood extract corresponding to 1½ c.c. blood. *e*. Action of 0·005 mgrm. pilocarpine.

TABLE I.

Exp.	Diagnosis.	Blood extr. in c.c.		Sensitiveness of the gut for pilocarpine in mgrm.	
A	Nephritis	5	No stimulation	0·005 mgrm.	++
B	*Nil*	4	,,	0·005 ,,	+
C	Trachei-bronchitis	4	,,	0·002 ,,	++
D	Lues (W. R. ++)	4	,,	0·002 ,,	++
E	*Nil*	4·5	,,	0·002 ,,	++
F	,,	5	,,	0·005 ,,	+
G	Lues (W. R. +)	5	,,	0·005 ,,	+
I	Nephritis	7	,,	0·005 ,,	++
K	Tuberculosis pulm.	10	,,	0·005 ,,	++
M	Tumour mediastini	12	,,	0·005 ,,	++

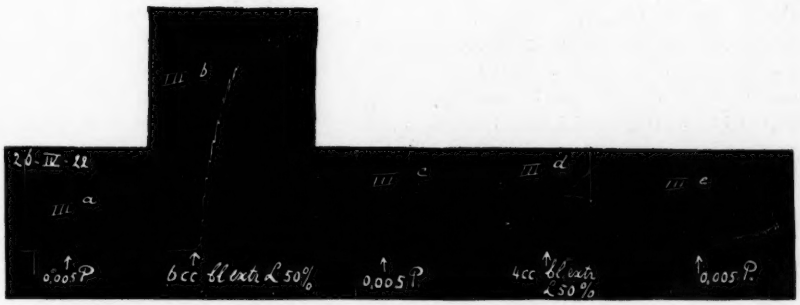

FIG. 3.—Action of blood extract from a severe case of urticaria. *a*. Action of 0·005 mgrm. pilocarpine. *b*. Action of 6 c.c. blood extract corresponding to 3 c.c. blood. *c*. Action of 0·005 mgrm. pilocarpine. *d*. Action of 4 c.c. blood extract corresponding to 2 c.c. blood. *e*. Action of 0·005mgrm. pilocarpine.

TABLE II.

Exp.	Diagnosis.	Stimulation substance in 1 litre blood corresponding to mgrm. of pilocarpine.
aI	Bronch. asthma	No stimulation.
aII	,,	± 5 mgrm.
aIII	,,	± 3·33 mgrm.
aIV	,,	± 3·33 ,,
aV	,,	± 2·5 ,,
aVI	,,	> 2·5 ,,
aVI	,,	< 5 ,,
aVII	,,	± 5 ,,
aVIII	,,	± 5 ,,
aIX	,,	± 2 ,,
H	Epilepsy	< 1·66 ,,
L	Urticaria	> 2·5 ,,
N	Migraine	> 2·5 ,,
Q	Urticaria	± 4 ,,

The finding of a toxic substance in human blood is not new. Many years ago it was shown that the blood of cases of epilepsy or eclampsia (Graf and Landsteiner, 1909) is more toxic for animals than normal blood. Recently Freund and Gottlieb (1921) found a poison in the blood of diseased animals and in certain human illnesses. The importance of the findings related in the present paper lies in the fact that a poison can be demonstrated in the blood of certain individuals during periods of apparently good health. So this toxic substance does not occur during an acute illness, but it seems to be an indication of a "pathological state," or of an "allergic disposition."

DISCUSSION.

The writers are quite well aware that the fact presented here, viz. the occurrence in blood extracts of twelve sufferers from allergic diseases of a toxic substance not present in normal blood extracts, although of considerable interest, by no means solves the problem of the mechanism of allergic conditions or asthmatic disposition. On the other hand it opens a new field for investigation where the clue to the problem might be found. Further work on the subject is in progress in this institute.

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APPLICATION OF THE ABSORPTION OF AGGLUTININ TEST TO THE SEROLOGICAL STUDY OF PNEUMOCOCCI.

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Received for publication October 13th, 1922.

THE pioneers of the serological classification of disease-producing pneumococci in New York (Avery, 1917) found that a number of strains titrated against Type II anti-serum exhibited agglutination, but only in lower dilutions and after more prolonged exposure than typical strains.

An analysis by myself of 53 cultures, derived not only from lobar pneumonia, but from a variety of other acute inflammatory conditions due to the pneumococcus, showed that the Type II group was very prevalent in London in the winter of 1919–20. The series included 16 strains obtained from cases of lobar pneumonia in adults, of which half were due to Type II infection and the remainder to Type I—a relative incidence of type corresponding to accepted statistics. Type II infection was responsible for the majority—73 per cent.—of the strains from all sources examined, a much higher relative incidence, but it was not found possible accurately to discriminate in every case by agglutinating titre and time response between typical and atypical strains of Type II, and the necessity for applying the further test of absorption of agglutinin in subsequent experiments became very apparent.

Considerable difficulty was experienced in 1919–20 in the production of a Type II anti-serum of good agglutinating power and scope—a difficulty also at first encountered in America. This was overcome after repeated trial of different strains by the discovery of a typical strain of good antigen content, isolated by blood culture *post mortem* from a fatal case of lobar pneumonia in an adult man.

The technique of the absorption test used in the experiments which form the subject of the present paper was as follows: 1 c.c. of an emulsion of the organism to be tested, of strength 8000 million cocci per c.c. (estimated by the opacity of the emulsion compared with a standard) was mixed with an equal bulk of serum diluted 1 in 5, and incubated for 2 hours in a water-bath at 37° C. in centrifuge tubes, the contents being shaken after 1 hour to secure complete admixture. The serum employed had agglutinating power up to dilutions of 1 in 80, and the above strength of emulsion of coccus was found by experiment to be sufficient to absorb completely the agglutinin present. The contents of the tubes were centrifugalised after incubation, 15 minutes usually sufficing to precipitate the suspension completely, and the clear serum removed by pipette and titrated against an emulsion of the homologous standard type coccus. The absence of any agglutination after incubation for 24 hours at 55° C. indicated total absorption of agglutinin and proved the strain to be a true homologue of the type coccus.

TABLE I.—*Serological Relationship of Type to Sub-type Pneumococcus Strains.*

A. Investigation of the effect of saturating a Sub-type I serum with corresponding Sub-type I and Type I coccus on the agglutinating power of the serum for its homologous coccus, with controls.

Conditions.—Saturation for 2 hours at 37° C. in the water-bath; centrifugalisation 15 minutes; clear, supernatant serum pipetted off and titrated against homologous coccus. Final titres read off after 12 hours' incubation at 55° C.

Stage I.—Saturation.	Stage II.—Titration of serum after saturation against an emulsion of the homologous sub-type coccus. Serum dilutions. 20.	40.	80.	160.	Result.
Sub-type coccus suspended in normal saline solution	–	–	–	–	No agglutination. Emulsion satisfactory.
Sub-type coccus emulsion in presence of normal serum	–	–	–	–	No agglutination by rabbit's normal serum.
Control agglutination of standard Type coccus by the sub-type serum	–	–	–	–	Absence of agglutination.
Control agglutination of sub-type coccus by homologous unsaturated serum	++	++	+	–	Serum titre determined.
Control agglutination after saturation with an indifferent coccus —Type II	++	++	+	–	Agglutinating power of serum unaltered.
Sub-type serum after saturation with homologous coccus	+	–	–	–	Almost complete absorption of agglutinin.
Sub-type serum saturated with standard Type coccus	++	++	+	–	Agglutinating power of serum unaltered.

B. Absorption of Sub-type agglutinin from standard Type serum by Sub-type and standard coccus. Titration against Sub-type coccus as in A.

Test agglutination of sub-type coccus by Type I serum	++	++	++	++	Serum titre determined.
Serum after saturation with sub-type coccus	–	–	–	–	Complete absorption of sub-type agglutinin.
Serum after saturation with standard Type I coccus	–	–	–	–	Complete absorption of sub-type agglutinin.

C. Effect on standard agglutinin of preliminary saturation with Sub-type coccus. Titration with standard coccus after saturation.

Control agglutination of Type I coccus by Type I serum	++	++	–	–	Serum titre determined.
After saturation with sub-type coccus	++	++	–	–	Agglutinating power of serum unaltered.

++ = Complete agglutination, clear fluid. + = Distinct agglutination, fluid not clear.
– = No agglutination.

The importance of controlling the experiment at each stage cannot be insisted upon too strongly, and is fully indicated in Table I. The behaviour of the homologous coccus and of a heterologous strain in the presence of the homologous serum before saturation of the latter, and likewise the behaviour of the unknown strains under investigation in the presence of rabbit's normal serum, have alike to be re-tested in each experiment, under conditions precisely similar to those to which the unknown strains are subjected. By this means the possibility of error due to variation in the behaviour of serums from time to time or spontaneous agglutination of an emulsion is excluded. The serums used have been prepared by rapid inoculation of rabbits, using the method described in a previous communication, viz. intravenous inoculation at intervals of 48 hours, with ascending doses of pneumococcus emulsion in phenolated saline solution; doses range from 2000 million to 10,000 million and upwards. Five or at most six inoculations usually suffice to produce a serum capable of completely agglutinating emulsions of the homologous coccus (strength 4000 million cocci per c.c.) in dilutions of 1 in 80, after 24 hours' incubation at 55° C. in the hot-air incubator. Such serums also agglutinate fully in dilution 1 in 40, and rather less completely to 1 in 80, within two hours in the water-bath at 37° C. Emulsions of coccus are obtained by suspending in 0·85 per cent. saline the surface growth on human blood legumin agar plates, after 12–16 hours' incubation, the suspensions being heated to 60° C. in the water-bath for 20 minutes to destroy autolysin. The emulsions are kept free from bacterial growth by addition of phenol to 0·5 per cent.

In the course of the present inquiry strains of pneumococci have been collected from many sources, embracing both hospital and general practice, and these may be considered fairly representative of the incidence of the several types. In establishing the identity of particular strains with true pneumococci, the accepted criteria of capsule formation, bile solubility and cultural characters have been rigidly observed, and results of plate cultures in many cases have been confirmed by mouse passage.

The absorption test has been applied to cocci of all three groups, with the result that the relationship of the standard Type II pneumococcus to its sub-types has been defined, and in addition the hitherto unsuspected existence of aberrant strains related to Types I and III has been discovered. The numerical frequency of each and their occurrence in disease is set out in Table III, based on an analysis of 200 strains obtained during 1920–22.

Sub-types of all three main types have been found responsible for lobar pneumonia.

The avoidance of confusion in the serological classification of pneumococci presents some difficulty. The position may be defined as follows: There are three or, with the possible addition of Lister's A strain, four pneumococcus types responsible for the majority of the most characteristic forms of pneumococcus infections in man—lobar pneumonia, empyema thoracis, meningitis and peritonitis. In addition an unknown number of strains exist, hereinafter described as sub-types, corresponding to each of the chief types. These sub-types possess in common the property of agglutinating with their corresponding "type" serum but not with serums homologous with other "types." A sub-type, although more closely related to its corresponding "type" than to other pneumococcal strains, is in other respects distinct from it. In the case of the sub-types of Type II, several strains serologically identical have been found in the case of each sub-type, but it is not yet possible to define the number of separate sub-types either of Type II or of Types I and III which exists. The serological characters of sub-type strains are described in greater detail below.

It is much to be regretted that the Rockefeller staff permitted themselves

to apply the loose term "Type IV" to the unclassified and ill-defined pneumococcus strains, which, although isolated from definite morbid sources, failed to agglutinate with any of their three representative serums. By so doing they have by implication closed the door for the admission of any other type of pneumococcus to the series. There seems strong reason for believing that Lister's Type A represents such an addition, and the question naturally arises how this organism may be recognised in future—if as Type V what strain shall be accepted as Type IV pneumococcus?

Table I illustrates the serological relationship of Type I pneumococcus and its allies. Type I is chosen as an example; the relationship of Types II and III to their sub-types appears to be similar.

The table shows that a sub-type coccus is agglutinated by and completely absorbs its own agglutinin, both from its own serum and the type-serum. Saturation of type-serum with sub-type coccus, however, in no way affects the agglutinating power of type-serum for type-coccus, or for other dissimilar sub-type strains. Apparently, therefore, the sub-type antigens can be removed piecemeal from the standard serum without in any way affecting its agglutinating power for the standard coccus or for other, dissimilar, sub-strains. Type I coccus is not agglutinated by a serum prepared against a "sub-type" coccus and has no power of absorption of sub-type agglutinin, but absorbs all the sub-type agglutinin as well as its homologous agglutinins from the standard serum, depriving this of all agglutinating power against either type or sub-type. These results are identical with those obtained by Castellani (1902), working with the typhoid-colon group, who proved that the agglutinins of any related strain can be removed from the standard serum by fractional absorption with their homologous strain, leaving the anti-bodies for similar strains and for the standard strain intact. No relationship between any of the sub-type strains of Type I has yet been discovered, each sub-type so far having exhibited specific antigenic qualities.

Of the seven strains agglutinated by Type III serum, investigated in the present inquiry, two were found incapable of absorbing the standard agglutinin. An anti-serum prepared against one of these was proved specific for its homologous coccus, and behaved in a manner strikingly comparable with the serums from sub-type I strains described above.

The technique employed has sharply delimited the standard Type II pneumococcus from the numerous strains which agglutinate with but do not absorb Type II serum. In view of the apparent complexity of this group the following statements based on the experiments may be made:

(1) The standard Type II pneumococcus contains an antigen, which is capable of eliciting in rabbits the formation of agglutinins, containing not only the factor capable of agglutinating the homologous standard coccus, but also rich in sub-agglutinins which react with atypical cocci. These atypical cocci are sharply differentiated from typical members of the group by failure to absorb the standard agglutinin.

(2) The higher the titre of the standard type anti-serum the richer and less specific appears to be its sub-agglutinin content. Titration experiments demonstrate that the titre for sub-strains is seldom so high as that for the truly homologous type-coccus.

TABLE II.—*Confirmation of Types, provisionally determined by Agglutination, by the Absorption Test.*

Reference.	Source.	Type-serum dilutions. Type I. 10.	20.	40.	80.	Type II. 10.	20.	40.	80.	Type III. 10.	20.	40.	80.	Normal Serum.	Inference.	Absorption of Type agglutinin.
Type I	Meningitis	++	++	—	—	—	—	—	—	—	—	—	—	—	Homologous	Complete.
Type II	Lobar pneumonia	—	—	—	—	++	++	+	—	—	—	—	—	—	No cross	Complete.
Type III	Lobar pneumonia	—	—	—	—	—	—	—	—	++	++	++	+	—	Agglutination	Complete.
49	Lobar pneumonia	++	+	—	—	—	—	—	—	—	—	—	—	—	Type I	Complete.
15	Lobar pneumonia	++	++	++	++	—	—	—	—	—	+	—	—	—	Type I	No absorption = sub-type.
52	Lobar pneumonia	—	—	—	—	++	—	—	—	—	—	—	—	—	Type II	Complete.
50	Chronic bronchitis	—	—	—	—	++	—	—	—	—	—	—	—	—	Type II	No absorption = sub-type.
48	Lobar pneumonia	—	—	—	—	—	—	—	—	++	+	+	—	—	Type III	Complete.
2	Acute mastoid	—	—	—	—	—	—	—	—	++	++	+	—	—	Type III	No absorption = sub-type.
7	Lobar pneumonia	++	++	++	+	++	++	++	++	—	—	—	—	—	Uncertain	Complete of Type II.

(3) Application of the absorption test to pneumococci belonging to the sub-types of Type II demonstrates a relationship between type and sub-type similar to that which exists between Types I and III and their subordinates. No anti-serum prepared against a sub-type II pneumococcus has possessed any agglutinating power for the standard Type II organism.

(4) The majority of strains of sub-type II obtained in the course of the inquiry were unrelated to one another, for no cross-absorption of agglutinin from sub-type serums occurred, but, in a few examples, identity, as proved by the absorption test, was found to exist. The similarity of these sub-types to Stillman's 2A and 2B has not been proved, nor have cultures of these strains, obtained from the National Collection of Type Cultures, exhibited agglutination either with any of the sub-type II serums prepared by myself, with my standard Type II serum, or with a Rockefeller Type II serum obtained direct from New York, but Griffith (1921) has recovered examples of both 2A and 2B from cases of lobar pneumonia in this country. It is possible that prolonged sub-cultivation *in vitro* has impaired the properties of the strains.

(5) An anti-serum was prepared by immunisation of rabbits against each of several of the sub-type II strains isolated in the course of the inquiry. Each serum was found to be possessed of agglutinating power, not only for other sub-strains, but also for strains previously exhibiting no reaction with any of the three standard sera. In addition these sub-type serums were found to agglutinate strains of organisms isolated from such trustworthy sources as lobar pneumonia sputums, before or immediately after crisis, which strains, although bile-soluble, differed in some of their characters from true pneumococci. These strains appear to be intermediate between pneumococci and the salivary streptococci.

(6) Furthermore, strains undoubtedly belonging to the group of salivary streptococci and culturally approximating to the descriptions of Schottmüller's *Streptococcus viridans*, show, when recently isolated from inflammatory exudates, feeble agglutination with Type II serums; but these strains differ markedly from the non-agglutinating and transitional forms of pneumococcus described above, in that they have vigorous agglutinogenic power when inoculated into rabbits. Serums with a titre of 1 in 640 can be prepared with ease by half-a-dozen graduated doses of such cocci at 48-hour intervals, affording the sharpest contrast with the pneumococcus group, which is notoriously of low agglutinogenic power. Two of these strains were selected for immunisation of rabbits, and the serums tested on a small group of twelve similar strains. One serum agglutinated its homologous coccus only, the other agglutinated two other strains as well as its homologue.

(7) So far as investigated, sub-type II pneumococci have lower agglutinogenic power than the standard organism. In serums prepared from them there is more rapid deterioration of agglutinin on keeping than in the case of standard serum, which maintains its agglutinating power remarkably well under all conditions of exposure, specimens under observation for two years having depreciated only 50 per cent. when stored in a cold and dark place. In addition, certain sub-type strains have been found, after repeated sub-cultivation, to lose the property of agglutinating with standard serum. As stated above, this observation seems to apply also to the cultures of Rockefeller Types

2A and 2B obtained from the National Collection of Type Cultures, mentioned in paragraph (4).

(8) From time to time well-authenticated strains of pneumococcus exhibit the phenomenon of agglutination in presence of both Type I and Type II serums but not in the presence of normal rabbits' serum; the absorption test has proved these invariably to be Type II pneumococci.

(9) These observations indicate that the agglutinins produced in the serum of rabbits in response to inoculation with Type II pneumococcus have much wider scope than those which result in the case of Types I and III.

TABLE III.—*Serological Analysis of Disease-producing Pneumoccoci*, 1920–22.

Disease.	No. of strains of the different types.								
	I.	I. Sub-types.	II.	II. Sub-types.	III.	III. Sub-types.	Un-classified.	Non-specific.	Total.
Lobar pneumonia .	20	4	21	5	3	2	9	6	70
Broncho-pneumonia, children . .	7	—	—	10	—	—	12	2	31
Bronchitis, adults .	8	—	—	4	—	—	8	3	23
Influenzal pneumonia, associated *B. Pfeiffer* . .	1	—	—	—	—	—	2	4	7
Empyema . .	14	—	—	—	—	—	—	4	18
Fauces, sore throat, pure culture . .	2	1	—	—	—	—	3	—	6
Nasal catarrh, pure culture. . .	7	—	—	4	1	—	12	4	28
Meningitis . .	4	—	—	—	—	—	—	4	8
Pericarditis (child) .	—	—	—	—	—	—	1	—	1
Middle-ear. Otitis .	1	—	—	—	1	—	1	1	4
Conjunctivitis . .	1	—	—	—	—	—	—	—	1
Peritonitis . .	3	—	—	—	—	—	—	—	3

Strains described as "unclassified" fail to agglutinate with any of the serums employed. "Non-specific" strains agglutinate indiscriminately with any pneumococcus anti-serum, but exhibit no absorption of specific agglutinin from any.

(10) It follows from the above observations that agglutination readings unsupported by a qualifying absorption test are an insufficient guide to type, and in not a few instances may be actually misleading as a therapeutic index. The protocol of an experiment in which agglutination results were corrected by the absorption test exemplifies this point (Table II).

Although carried out entirely independently, the above results are substantially identical with those obtained by Avery (1915) in his investigation of 10 strains of aberrant Type II pneumococci. Unfortunately, my experiments lack the complete proof which virulence tests would have afforded and

which Avery was able to supply. His study of the protective power of anti-serums, prepared against members of the sub-types of Type II, confirms and extends the results arrived at by means of agglutination and absorption tests. Of particular importance is his observation that saturation of the standard serum by its homologous coccus removes all the agglutinins and protective bodies, while absorption by a related sub-type strain does not materially affect these.

It may not be altogether out of place to review here the significance of the added complexity of type revealed by the experiments summarised above. Avery clearly demonstrated by immunological methods that the standard Type II serum has protective power against organisms of the two sub-groups 2A and 2B, although not against the mixed coterie of strains described by him as Group 2X. The serological affinities between the typical and atypical members of Types I and III, identified and described by me, appear to be of the same order as those which exist between Type II and its sub-types A and B.

If this be confirmed by subsequent experiment, it may well be that infection by sub-types I and III is, in a similar manner, amenable to treatment with corresponding standard serums.

The phenomenon of macroscopic agglutination is a two-phase reaction :

(*a*) Conjugation of coccus and agglutinin.

(*b*) Precipitation in presence of an electrolyte.

Theoretically it is possible that the agglutination reaction may at times be incomplete, in the sense that stage (*b*) does not ensue. To test this hypothesis type-serums have been saturated with non-agglutinating (unclassified) strains of pneumococcus in order to ascertain if absorption of agglutinins occurs such as might indicate an occult relationship to type. In every case, using each of the three standard serums, the results have been negative, *i.e.* phases (*a*) and (*b*) were both absent.

The numerous non-agglutinating strains of pneumococcus variously described as Group IV and sometimes improperly as Type IV are, in my experience, conspicuously deficient in antigen for rabbits ; in one example in which a series of doses of killed vaccine were followed by inoculation with living culture, resulting in a septicæmia of ten days' duration, only the feeblest agglutinating power developed, even when undiluted serum was employed.

The experiments described have been carried out in the Research Laboratory of St. Bartholomew's Hospital by permission of Sir Frederick Andrewes, to whom, and to Dr. H. M. Gordon, Bacteriologist to the Hospital, I am greatly indebted for much practical advice and help.

The work was undertaken with the assistance of the Medical Research Council, to whom my grateful thanks are due.

CONCLUSIONS.

(1) Agglutination by specific pneumococcal serums is not invariably sufficient for recognition of type. The absorption of agglutinin test affords satisfactory confirmation.

(2) The use of the absorption test has led to the identification of sub-types of Type I and Type III pneumococcus comparable to the sub-types 2A and 2B of Type II already differentiated by Avery.

(3) Sub-type strains exhibit limited specificity within the group to which they belong; in no case has a serum prepared against a sub-type pneumococcus agglutinated the standard type strain.

(4) The serum of rabbits inoculated with the standard Type II pneumococcus is particularly rich in subordinate agglutinins. This property is shared by sub-type 2 strains in general, since the sub-type serums were found to agglutinate pneumococcus strains which had failed to react with any of the three standard serums. By this means a degree of serological relationship between the Type II group and salivary streptococci of the *S. viridans* group has been demonstrated. Salivary streptococci are sharply differentiated from pneumococci by their vigorous agglutinogenic properties.

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THE INFLUENCE OF THE QUALITY OF THE MEAT USED UPON THE REACTION CURVE OF A NUTRIENT MEDIUM.*

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Received for publication July 26th, 1922.

PREVIOUS investigations into the type of meat most suitable for bacterial metabolism have referred chiefly to the development of the maximum toxigenic power of *B. diphtheriæ* in nutrient broth. The presence of fermentable substances was by some thought undesirable owing to the production of acid. Th. Smith (1895), however, while endeavouring to avoid "muscle sugar," thought that small quantities of glucose were beneficial.

In the present paper the quantity of glucose in meat is determined and the effect of different meat samples upon the reaction curve of the medium during growth of *B. diphtheriæ*.

THE AMOUNT OF GLUCOSE IN MEAT INFUSIONS.

Cadeac and Maignon (1902) published sets of analyses of various muscle tissues for carbohydrates, but an examination of their results does not allow one to draw any very certain conclusions. They used Fehling's solution to

* A Report to the Medical Research Council.

estimate the sugar in the bouillon "après défécation." It is difficult to eliminate the extent to which nitrogenous substances were responsible for reduction. They found the greatest concentration of glucose in heart muscle. Smith (1895) estimated that the glucose in meat varied from a trace to 0·3 per cent. Fresh meat contained less than that which has been allowed to stand. This statement is remarkable in view of the modern idea of glycolysis, yet the present experiments appear to bear out what Smith asserted.

There is little other information of value regarding the concentration of glucose in muscle tissue.

In a survey of the methods which would be available for the estimation of amounts of glucose of the order supposed to be in bouillon that of Benedict was chosen. Previous experience with the method of Dufau and Patein of precipitation with mercuric nitrate had shown that this removed quantitatively such substances as creatinine, which might in the subsequent digestion give a colour with alkaline picrate solutions. I have further also found that glucose was quantitatively recovered from urines to which it was added after precipitation with this reagent.

For the purpose of the present experiments, weighed quantities of meats freed from fats as completely as possible and finely minced were placed in somewhat more than their weight of cold water. The mixture was raised slowly to 80°C. It was then filtered through paper and allowed to drain well and the amount of filtrate measured. An aliquot portion of the filtrate was used for analysis, the volume being referred to the amount of meat originally used. The procedure was essentially that described by Benedict. The picric acid used had been recrystallised three times by Folin's method. The meats used were: (1) English beef, freshly killed; (2) imported beef; (3) bullock's heart, English freshly killed.

The following results were obtained:

English beef . .	0·039 per cent. glucose.
Foreign beef . .	0·073 „ „ „
Heart . . .	0·0 „ „ „

In order to see if the colours produced by the alkaline picrate were due to fermentable substances, the meat infusion in each case was heated with fresh bakers' yeast at 37°C. for 12 hours, and the mixture treated with mercuric nitrate and picric acid as before. No colour developed in any of the samples. The colour was therefore due in the first instance to a fermentable substance.

The results with heart muscle were so contrary to expectations that the whole experiment was repeated, using pig's heart. In addition a portion of bullock's heart was obtained from the slaughter-house and worked up in less than an hour from the time the animal was killed.

The following results were obtained:

English beef, freshly killed . .	0·088 per cent.
Foreign beef	0·16 „ „
Veal	0·14 „ „
Heart (pig's)	0·0 „ „
Heart (bullock's fresh) . . .	0·0 „ „

In this set of experiments the meat infusion was fermented with yeast, and in no instance was it possible to obtain a colour with alkaline picrate.

THE EFFECT OF DIFFERENT SAMPLES OF MEAT UPON THE REACTION CURVE OF A MEDIUM.

These experiments were carried out to determine the difference in effect of freshly killed English meat and foreign chilled meat. Several sets of experiments were carried out, but only one is reported here.

The experiments were made in test-tubes. Two per cent. of peptone and 0·5 per cent. of sodium chloride were added to the meat infusion, made by heating meat with twice its weight of water to 80° C., and filtering.

After adjusting to particular reactions and sterilising, the media were checked with the hydrogen electrode. As will be seen the differences in absolute reaction were very great, due partly, it would appear, to the quality of the meat and partly to the quality of the peptone. All tubes were inoculated with a rapidly growing culture of *B. diphtheriæ*, and a tube taken on each of the specific days for a hydrogen ion concentration determination.

One sees in the present experiment the profound effect obtained by starting the fermentation with an initial hydrogen ion concentration in the neighbourhood of neutrality. Where the initial concentration was pH 7·1 the final reaction became more acid than pH 5·6. pH 7·5 would seem to be the limiting concentration for reversal, and statements of other workers appear to confirm this.

The results are given in Table I.

TABLE I.

Days.	Foreign beef and Parke Davis' peptone.	English beef and Parke Davis' peptone.	Foreign beef and Morson's peptone.	English beef and Morson's peptone.	Foreign beef and Witte's peptone.	English beef and Witte's peptone.
0	7·6	7·6	7·7	7·5	7·3	7·1
4	7·0	6·6	6·9	7·1	6·6	7·1
7	6·9	6·5	6·9	6·6	6·1	5·6
11	6·9	6·5	6·9	6·9	6·2	5·6
14	6·9	6·8	6·8	6·9	6·2	5·6
18	+8·0	7·6	+8·0	8·2	6·9	−5·6
25	+8·0	7·2	+8·0	+8·0	6·7	−5·6

The results as given in the above table do not lead to perfectly straightforward conclusions. The initial reaction is obviously not an all-important factor, for while the more alkaline media do reverse, one at least, made with English beef and Parke Davis' peptone, had only reached a pH of 7·6 on the eighteenth day. On the other hand, a medium made with English beef and Morson's peptone, which only had an initial alkalinity of pH 7·5, reversed as quickly and as far as the companion medium made with foreign meat, which started with an alkalinity of pH 7·7. These results confirm what Hartley has found in his study of peptones, viz. that each peptone has individual qualities which must be taken into consideration if the best possible use is to be made of them.

That the quality of the meat infusion may have a very serious effect on the course of a bacterial growth is shown from another experiment in which Parke Davis' and Morson's peptones were combined with two infusions and the

cultures of *B. diphtheriæ* allowed to incubate for two weeks. The results were as follows:

	Initial pH.	Final pH.
Parke Davis and foreign meat	6·85	7·82
Parke Davis and English meat	6·79	6·55
Morson and foreign meat	6·89	8·39
Morson and English meat	6·74	6·74

Here one sees that there was no reversal when the English meat infusion was employed, while the results were quite satisfactory with the chilled samples.

CONCLUSION.

Chilled foreign meat contains more glucose than fresh English meat, but nevertheless the reversal which occurs in the successful production of toxin takes place more constantly when chilled beef is used.

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ON VITAMIN UNDERFEEDING.

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Received for publication November 13th, 1922.

THE relations of vitamins to dietetics present two different aspects, one of which has so far been completely neglected. Up to now only the two extreme conditions have received consideration: the effects of a diet from which one or more vitamins are completely or almost completely excluded, and which eventually leads to disease, is contrasted with what is called the "normal" condition resulting from a diet rich in vitamins. These studies have been of great importance in identifying the presence of vitamins in the various articles of food. They have, however, led to a misconception of their mode of action, and of the ætiology of the so-called deficiency diseases. And they have failed to recognise that there exists a condition of "vitamin underfeeding"—as distinct from vitamin deficiency—where the supply of vitamins, though adequate to prevent obvious ill-health and to enable the animal to grow and breed, does not permit the organism to reach its full development. As is to be expected, pregnancy, lactation and infancy are the periods in which the ill-effects of vitamin underfeeding are most likely to make themselves felt, and these periods will be particularly dealt with in this paper. But before considering the importance of this condition it is necessary to subject the

prevalent vitamin conception to a brief criticism. It may be added at once that this criticism in no way detracts from the importance of the vitamins in dietetics, but on the contrary emphasises it.

A CRITICISM OF THE PREVALENT VITAMIN CONCEPTION.

The prevalent conception of vitamins is based on the fundamental assumption that a supply of vitamins is necessary for the life of every cell. In what way they act is admittedly not understood. It is clear that they are not a source of energy. It is stated that very minute amounts are sufficient to ensure a normal cell metabolism, and they are not supposed to have any positive action, so that their existence can be recognised only through the effect produced by their absence over prolonged periods. If their supply is withheld diseases develop which are held to be specific for vitamin deficiency. These eventually kill the animal, but according to the orthodox vitamin conception they do so in the absence of pathological lesions sufficiently severe to account for the death of the animal. Death is supposed to be the result of a failure in the vital activities of the general mass of cells in the body.

This view introduces a new pathological conception of the ætiology of disease, and those diseases in which vitamin deficiency is held to be the only ætiological factor have therefore been called "deficiency diseases." Conversely, the occurrence of such diseases in man or animals is taken as indicating a vitamin deficiency as the only ætiological factor. And the absence of these specific diseases in man and animals living on ordinary unrestricted and freely chosen dietaries has led to the comfortable belief that any freely chosen diet is adequate from the point of view of vitamin supply, and that "we need not bother about vitamins."

This conception does not necessarily follow from the facts observed and involves a number of assumptions. The whole evidence of the existence of vitamins is based on the fact that a highly organised animal (a mammal or a bird) dies when vitamins are withheld from the diet. As we pointed out in previous papers (Cramer, Drew and Mottram, 1921, 1922), this observation shows that the organism as a whole requires a supply of vitamins, but it does not necessarily follow from it that all the cells of such an animal require vitamins. And since the adrenal gland hypertrophies in vitamin deficiency, further subsidiary assumptions would be necessary to avoid the conclusion that the cells of the adrenal gland do not require a supply of vitamins, but can maintain themselves and actually grow in their absence. Further observations on the growth of mammalian tissue *in vitro* do not agree with the contention that all the cells of a mammal require vitamins. Drew (1922) has been able to grow such cells in a saline medium for an indefinite length of time if a watery extract of embryo was added to the saline medium. Adult tissues were found to contain much less of the substance or substances capable of maintaining growth *in vitro*. Feeding experiments have failed to show that embryos are particularly rich in vitamins. Moreover, the active substance present in the embryo extract differs from vitamins in being very thermolabile; it is inactivated by heating the extract for 10 minutes to 60° centigrade. Lastly embryo extract cannot be replaced by a yeast extract, which has no effect at all in promoting growth of isolated cells *in vitro*.

When we published our first criticism of the current vitamin conception it was still firmly believed that all forms of cell life, even the lowest, such as bacteria, yeast cells and plant cells, required a supply of vitamins. This view was so firmly held that methods had actually been devised to test for the presence of vitamin B, and estimate it quantitatively, by its effect on the growth of yeast cells. We know now that yeast cells can synthetise vitamin B, and that plant cells can synthetise vitamin A. These recent observations would therefore necessitate a modification of the original contention that all cells require vitamins. Quite recently the curative effect of light in rickets has led to the further assumption that even in a highly organised animal some cells can synthetise vitamin A under the influence of light and pass it into the circulation for the benefit of other cells. It does not seem to have been noticed that this new assumption involves a reversal of the original contention. It may be added that the available experimental evidence fails to confirm this assumption. So far no convincing demonstration has ever been given of a failure in the vital activities of the general mass of cells in animals suffering from a vitamin deficiency. In our observations we have equally failed to get an indication of such a general wide-spread failure. Histologically the finer cytoplasmic structure of the cells remains preserved even in the last stages of vitamin deficiency and no definite nuclear changes could be detected. Physiologically it might be expected that a general failure would make itself felt in the sensitiveness of the sympathetic nervous system. But in experiments (unpublished) carried out with Dr. Samson Wright, it was found that intestinal strips from rats in the very last stage of vitamin deficiency responded as actively to adrenalin as intestinal strips from normal rats. In fact the former were more sensitive than the latter. In view of all this evidence it seems advisable to go back to the experimental facts which established the existence of vitamins, and to realise that these prove only that vitamins are necessary for the life of a highly organised animal as a whole. There is no evidence that the ill-health occasioned by their absence is due to a general wide-spread failure of the vital activities of every individual cell.

In searching for an explanation of this ill-health (Cramer, Drew and Mottram, 1922), we have found, on the contrary, that there are definite lesions in certain tissues, which are specific for each vitamin and which produce specific effects in the organism. There is atrophy of lymphoid tissue and, as a result, marasmus in vitamin B deficiency; there is a thrombopenia and, with it, increased susceptibility to infection in vitamin A deficiency. These same lesions can be produced by agencies other than vitamin deficiency, such as the radiations of X-rays or radium, and when these lesions are thus produced, there is the same effect on the organism as a whole. In other words, the so-called " deficiency diseases " are, like any other disease, the result of specific pathological lesions which can be produced by a variety of agencies of which the deficiency in vitamins is only one.

It follows from this that the production of a disease by withholding a vitamin does not exclude the possibility that the same disease might not be produced by other agencies even in the presence of this vitamin. In the light of this conception the controversy concerning the ætiology of certain so-called

deficiency diseases assumes a new aspect, and the discrepancies between clinical experience and experimental observations disappear.

Lastly, there is the tenet of the current vitamin theory that these substances have no positive action. Our observations (Mottram, Cramer and Drew, 1922) have shown that vitamins produce an immediate stimulating effect on the processes of digestion and assimilation, and that this effect takes place even in a normal organism which has not previously been subjected to any vitamin deficiency. These observations agree with the findings of other observers (Voegtlin and Myers, 1919, Anrep and Drummond, 1921), who showed that vitamin preparations stimulate the activity of the digestive glands and are capable of producing a flow of secretion from them. The important conclusion follows that the various articles of food contain substances capable of stimulating digestion and assimilation, and they contain these substances, which might be called "food hormones," in varying quantities. An analogy may be drawn between the atrophic changes in specific tissues such as the atrophy of lymphoid tissue when the constant stimulus of the food hormone, water-soluble B, is withheld over prolonged periods, and the atrophic changes which take place, for instance, in the uterus after removal of the ovary, and which are generally attributed to the absence of an ovarian hormone. That the complete or almost complete absence of these "food hormones" leads to disease reveals only the negative aspect of their significance. There is also the no less important positive aspect that their presence has a stimulating action on specific functions, and that the different dietaries will differ in the positive stimulating action according to the supply of vitamins they contain, and that the aim must be to ensure a maximum supply.

THE EFFECT OF VITAMIN UNDERFEEDING ON YOUNG RATS.

The significance of this conclusion is exemplified in a most striking manner by the difference between a stock of rats kept on our ordinary laboratory diet and a stock of rats receiving the same diet with an ample supply of vitamins. In this laboratory large numbers of rats have been kept for years on a regular diet of bread and water supplemented with boiled maize and rice. On this natural diet they breed freely, they grow, they do not suffer from epidemics, so that the laboratory stock has replenished itself for years with the occasional introduction of fresh stock. Occasionally a litter would fail to grow and sometimes a case of spontaneous rickets has been observed, but such cases are exceptions. On the whole these animals were to all appearances normal healthy animals, which compared favourably with many of the rats obtained from breeders. I had noticed, however, that though these animals grow, their rate of growth is much slower than that given by Donaldson as a standard in his book on the rat. But as soon as material rich in vitamin, such as marmite and cod liver oil, was added to the laboratory diet the animals grew much more rapidly, although even then they did not always attain the standard rate given by Donaldson. The differences in the size of rats of equal age kept on the laboratory diet alone and on the same diet supplemented with vitamin-rich material are remarkable. Details of such observations have been given in a previous paper. These data show that a rat kept on a vitamin-rich diet may

have double the weight of an animal kept on the laboratory diet alone and have a corresponding increase in length. The functional activity of the lymphoid tissue in the former case is considerably increased and there are other differences which need not be detailed here.

Our laboratory diet of bread, rice and maize has, therefore, for years enabled our rat population to lead a normal vegetative and reproductive existence. This diet, which is not very rich in vitamins, contained nevertheless sufficient vitamins for that purpose, but not sufficient to ensure the full development of which the rat is capable. It is more than probable that large classes of civilised mankind live on diets of a correspondingly restricted vitamin content, adequate to prevent obvious ill-health but not adequate to ensure the maximum development. It may be added that our laboratory diet is also poor in calcium. This dietetic fault is, of course, not corrected by the addition of vitamins.

The fact that intestinal digestion is subject to the influence of "food hormones" is a fact of considerable importance which is not yet sufficiently recognised. It is likely to be of particular importance in such a condition as pregnancy where gastro-intestinal disturbances are so easily elicited. It is equally important for the nutrition of the infant. Clinical experience has already shown that in certain types of marasmus in infants, where in spite of careful feeding and treatment the child fails to put on weight, the administration of vitamins in the shape of cod liver oil, malt and orange-juice is capable of rapidly relieving the condition.

VITAMIN UNDERFEEDING IN PREGNANCY AND LACTATION.

A study of the work on the metabolism of pregnancy—a detailed account of which will be found in a chapter by Lochhead and Cramer in the forthcoming new edition of the 'Physiology of Reproduction,' edited by Dr. F. H. A. Marshall—reveals as the outstanding change in the pregnant organism that it acquires the power to retain and store nitrogen over and above the needs of the fœtus. This process is probably a preparation for the purpose of laying down a store for the period of lactation. Now the fact that an animal increases in size and weight the more rapidly vitamins are supplied to it means in terms of metabolism that vitamins favour the retention of nitrogen. It is therefore to be expected that in pregnancy the characteristic retention of nitrogen should be dependent also on the supply of vitamins. The vitamin factor was unknown when the experimental observations on the nitrogen metabolism in pregnancy were carried out and had therefore not received any consideration. But the data given in the various papers are sufficient to show that the characteristic nitrogen retention of pregnancy is indeed dependent on the vitamin content of the diet. Thus the earliest observations on dogs (by Hagemann and by Jägeroos) showed a loss of nitrogen during pregnancy on a high nitrogen intake, while with a lower nitrogen intake Bar obtained a retention of nitrogen. The diet in the earlier experiments was either lean meat alone, or supplemented by cane sugar—a diet very poor in vitamins. Bar gave meat together with bread and animal fat—a diet richer though by no means abundant in vitamins. The pregnant woman observed by

Hoffström, who showed a remarkably high nitrogen retention, lived on a very varied and vitamin-rich diet, in which milk and butter, for instance, figure largely.

In order to test this important point directly, fresh observations have been carried out on a number of female rats. These rats were kept together with male rats on the laboratory diet of bread and water, maize and rice. In some cases this diet was supplemented by marmite, as a source of the water-soluble vitamin, and cod liver oil, as a source of the fat-soluble vitamin. The weight was taken once a week and as soon as the animal had littered. The litter was weighed separately. The period of gestation was taken from our observations as 23 days and the day of conception calculated accordingly. In this way it was possible to determine whether the mother had lost or gained during pregnancy. In some cases the same animal was observed first on the one diet, then on the other.

The results are given in the following table:

I. *Laboratory Diet + Vitamins A and B.*

No. of rat.	No. of successive pregnancy.	Wt. of mother in grms.		Loss or gain of mother in grms.			Litter.			Interval in weeks between end of lactation and succeeding conception.
		At conception.	After littering.	During pregnancy.	During lactation.	Total.	Total wt. in grms.	No. of young.	Average wt. of single young.	
223	3rd	215	220	+ 5	± 0	+ 5	45	7	6·5	1
—	4th	225	250	+ 25	− 20	+ 5	45	8	5·6	3
221	2nd	140	165	+ 25	Not determined.	—	33	5	6·6	1
222	—	170	230	+ 60	—	—	45	8	5·6	Not known.
265	1st	160	185	+ 25	+ 10	+ 15	24	4	6·0	Do.
—	2nd	205	230	+ 25	− 25	± 0	34	6	5·7	12
II. *Laboratory Diet alone.*										
—	1st	210	220	+ 10	− 25	− 15	50	9	5·5	Not known.
223	2nd	215	230	+ 15	− 45	− 30	40	8	5·0	4
—	6th	220	230	+ 10	− 40	− 30	48	6	8·0	3
221	1st	145	145	± 0	− 25	− 25	60	9	6·7	Not known.
266	—	220	220	± 0	Not determined.	—	Litter partly eaten.			Do.

It should be noted that the observations were made on rats which had not yet attained their full weight. The table shows that on the vitamin-rich diet the rats complete pregnancy as a rule with a considerable gain in weight. On the laboratory diet alone the gain in weight is absent or much less. The difference becomes more marked in the period of lactation. Lactation imposes an even heavier drain on the mother, so far as energy requirements are concerned, than pregnancy. For, as Murlin has shown, the energy requirements of the newborn are much higher than those of the embryo immediately before birth. This is due to the fact that the newborn requires additional

energy to maintain its body temperature against a cold environment. We find accordingly a considerable loss of weight on both diets. But this loss is slight on the laboratory diet enriched with vitamins, while it is considerable on the laboratory diet alone. Taking periods of pregnancy and lactation together, one finds that the weight of the mother remains practically unaltered when she receives the laboratory diet enriched with vitamins, but suffers a considerable loss of weight on the laboratory diet alone. This may be of serious import when pregnancies rapidly succeed each other, so that a new pregnancy begins before the period of lactation of the preceding pregnancy is completed. In this series of observations care has been taken to let at least one week elapse between the end of the period of lactation and the beginning of the next pregnancy.

VITAMIN-UNDERFEEDING IN INFANTS.

The most important aspect of this problem refers, not to the mother, but to the offspring. The vitamin content of the milk is dependent on the vitamin supply to the mother. There is a difference between two litters, of which one is suckled by a mother which receives the laboratory diet alone, while the other is suckled by a mother kept on the laboratory diet enriched with vitamins. This difference becomes greater if the dietetic differences are maintained through successive pregnancies. This difference manifests itself not only in the rate of growth, but also in the general development, such as, for instance, the growth of the fur. And this difference persists and becomes more marked when after weaning the two litters the difference in the two diets is maintained. There is then a very distinct difference in the rate of growth. This is the most obvious effect, but the effect in growth is only a crude manifestation. More essential and more important differences exist between two such litters, although there is nothing apart from the difference in weight and size to indicate those differences, either macroscopically or microscopically. Young rats kept on the laboratory diet alone look perfectly healthy and show no obvious signs of disease. But a significant difference reveals itself when rats from two such litters are now deprived of the fat-soluble vitamin. Animals derived from a litter kept on the laboratory diet alone will at once stop growing, and within 8–10 weeks at the latest develop the typical eye lesion and look ill and emaciated. But if the animals have since birth been kept on the laboratory diet enriched with vitamins they may continue to grow for a considerable time. Their growth in the absence of the fat-soluble vitamin may even be more rapid than that of normal rats receiving the laboratory diet. They look perfectly well and healthy for three or four months, and only develop the typical eye lesion two or three months later than the rats from the other litter. Two of these rats were kept on the completely vitamin A free diet for nine months before the eye lesion developed, and the only obvious evidence of the absence of the fat-soluble vitamin was that they did not attain their full weight, and in that eventually they did not look quite so sleek as a normal animal. Fig. 1 represents such an experiment. Three rats from a litter ("Y"), kept on the laboratory diet alone, and three rats of the same age from another litter ("Z"), kept on the laboratory diet plus vitamins, were placed together in one cage. They were fed on the vitamin-free basal ration, to which

the water-soluble vitamin was added in the form of marmite. The six rats lived therefore under exactly the same conditions. The rats from litter "Y" ceased growing immediately. Two developed keratomalacia within 8–9 weeks and died; the third died of pneumonia in the ninth week. The rats from litter "Z" continued to grow actively, looked well, and did not develop the eye lesion within the twenty weeks during which the experiment was continued, although they were living in the same cage as the rats with the eye infection and were therefore exposed to the same extraneous risks of the infection. The animals which show the least resistance to the infection are those which had been subjected to vitamin underfeeding since birth. It might be argued that this difference is simply due to a storage of the fat-soluble vitamin A in the tissues of the animal during infancy when they receive a vitamin-rich diet. But such an explanation does not account for the fact that such animals continue to grow as quickly when the vitamins are withheld as animals which, after having been subjected to vitamin underfeeding since birth, are placed, when half-grown, on a vitamin-rich diet. In fact, sometimes animals from a "vitamin-rich stock" may grow when placed on a vitamin-free diet even more rapidly than animals from a "vitamin-underfed stock" when placed on a vitamin-rich diet.

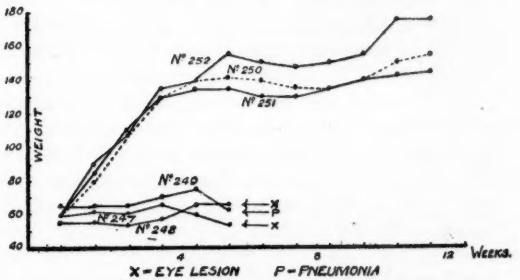

FIG. 1.—Weight curves of six rats from two litters kept in the same cage on a diet free from vitamin A (basal ration and salt mixture and marmite). Rats No. 247, 248, 249 belonged to one litter taken from a vitamin-underfed stock and had themselves been kept since birth on the routine laboratory diet. Rats 250, 251 and 252 belonged to another litter taken from a stock kept on the routine laboratory diet plus marmite and cod liver oil and had themselves received this diet since birth. The curves illustrate the delayed effect of vitamin under-feeding in early life.

Data of another such experiment with similar results have been given in a recent paper (Cramer, Drew and Mottram, 1922). In that paper it was shown that the effect of withholding the fat-soluble vitamin A is to reduce the number of blood-platelets by 60 to 80 per cent. of the normal. Associated with this thrombopenia there is a diminution in the resistance of the animal to certain types of bacterial infections. Rats which have received an abundant supply of vitamins since birth do not undergo this reduction in the number of platelets as readily and as quickly as animals from a "vitamin-underfed stock," and also retain their resistance to those infections over a longer period.

These observations should not be taken as implying a criticism of the methods of the so-called quantitative estimation of vitamin A, although they probably account for many of the discordant statements which have been made concerning the content of certain foodstuffs in this vitamin. Our curves show that though the degree of sensitiveness to a deficiency in this vitamin varies widely in normal animals taken at random, a very constant degree of sensitiveness can be obtained if the dietetic conditions to which the animal

has been subjected since birth have been kept constant, and can be varied at will by varying those preliminary dietetic conditions. The point we wish to emphasise is that the negative conception of the mode of action of vitamins should be replaced by the positive conception that these substances have specific positive, stimulating actions, and that the varying degree in which an organism has been subjected to the action of these food hormones, especially during infancy, produces lasting differences in certain tissues. These differences do not necessarily manifest themselves in obvious ill-health: they exist in a latent form between apparently normal animals, using the word "normal" in the sense in which it is usually used as indicating the absence of obvious disease.

SUMMARY.

Observations have been made on a stock of rats which have been kept through many generations on a natural diet, the vitamin content of which, though restricted, is adequate to enable them to grow and breed and to prevent the occurrence of obvious ill-health. A comparison of rats from these stocks with animals from a stock fed on the same diet supplemented with an abundant supply of vitamins shows that there is such a condition as "vitamin underfeeding" and that it may occur on a natural diet. Such vitamin underfeeding does not lead to any obvious ill-health: the animals are in appearance normal, healthy animals. But vitamin underfeeding, especially if it has occurred in infancy, impresses itself upon the organism as a lasting weakness which only manifests itself when the organism is exposed to a strain. Hence the importance of insuring an abundant supply of vitamins in the food, especially to the pregnant and lactating mother and to the growing child.

The fact that vitamins have a positive, stimulating drug-like action and thus act as food hormones is put forward to replace the prevalent conception of their mode of action.

These conclusions open up a social aspect of the vitamin problem which has hitherto not been recognised, but which is at least as important as the actual production of diseases by a severe vitamin deficiency. They suggest that the physical make-up of a community is determined largely by the ease and regularity with which an abundant supply of vitamins is secured to the pregnant and nursing mother and to the growing child.

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THE LABORATORY PREPARATION OF A PURIFIED HÆMATOXYLIN.

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Received for publication November 3rd, 1922.

It is well known that many of the commercial preparations of hæmatoxylin have proved unsatisfactory as histological stains. Indeed, since the war, there has been no good hæmatoxylin on the market. It was found that the most unsatisfactory feature of all the commercial samples was a diffuse pink staining over the entire specimen, which interfered with the black staining of the hæmatoxylin when used by Heidenhain's method. In addition to the admixture with the pink compound, it was found that the hæmatoxylins were too fully oxidised, which apparently interferes with their staining properties. The ideal preparation seems to be a solution containing the hæmatoxylin as a leuco-base which is oxidised in the tissues themselves.

Hæmatoxylin may be reduced in alcoholic solution by means of finely divided zinc dust. The commercial hæmatoxylin is dissolved in absolute alcohol to form a 5 per cent. solution, five times the weight of zinc dust is added to the solution, and the whole boiled gently for two hours in a flask fitted with a reflux condenser. Not all specimens of zinc were found suitable, as much of that on the market is far too coarse, and is in fact zinc filings and not dust. The zinc should be in as finely divided a state as possible. During the boiling the hæmatoxylin loses its colour, the pink staining substance is thrown out of solution, and is adsorbed by the zinc. After boiling for two hours the hot solution is rapidly filtered through paper into a clean dry bottle. This filtration is best carried out either in an atmosphere of hydrogen or of coal gas. The solution should come through the filter either colourless or with only a faint yellow tint. It is convenient so to arrange the amount of hæmatoyxlin that the final filtered solution is of about 5 per cent. strength, as this can be readily diluted to form the ordinary laboratory stock solutions. Solutions made up from this reduced hæmatoxylin are nearly colourless at first, but stain perfectly well, iron-hæmatoxylin preparations showing clean jet black pictures, with no diffuse staining whatever. Ehrlich's acid hæmatoxylin made up in the usual way with this purified stain is very good. For Weigert's iron hæmatoxylin dilute the acid ferric chloride solution with an equal volume or more of 0·5 per cent. hydrochloric acid in water. Five volumes of this diluted iron solution is added to five volumes of 0·5 per cent. hæmatoxylin in 95 per cent. alcohol.

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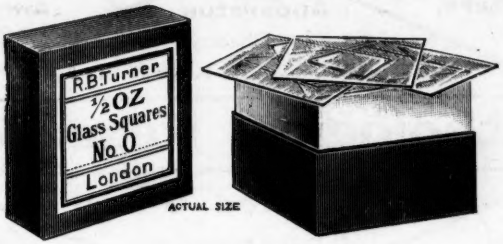

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